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Award Number: DAMD17-98-1-8465

TITLE: Tumor Specific Regulation of C-CAM Cell Adhesion Molecule in Prostate Cancer Carcinogenesis

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REPORT DATE: March 2004

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	March 2004	Final (1 Aug 1998 - 28 Feb 2004)

4. TITLE AND SUBTITLE

Tumor Specific Regulation of C-CAM Cell Adhesion Molecule in Prostate Cancer Carcinogenesis

5. FUNDING NUMBERS

DAMD17-98-1-8465

6. AUTHOR(S)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

Original contains color plates: ALL DTIC reproductions will be in black and white

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Down-regulation of carcinoembryonic antigen-related cell adhesion molecule (CEACAM1) tumor suppressor gene expression is common in several malignancies including prostate. The mechanism that mediates this down-regulation is not known. We propose to elucidate the mechanism of loss of CEACAM1 tumor suppressor expression in prostate cancer. We found that down-regulation of CEACAM1 expression in prostate tumors is mainly due to transcriptional down-regulation of CEACAM1 gene. We have identified two transcription factors, i.e. AP-2 and androgen receptor, that are involved in the up-regulation of CEACAM1 gene expression and one transcription repressor, i.e. Sp2, that specifically down-regulates CEACAM1 promoter activity in tumor cells. The identification of Sp2 as a transcriptional suppressor of CEACAM1 gene is a novel finding. We found that Sp2 represses CEACAM1 gene expression by recruiting histone deacetylase activity to the CEACAM1 promoter. Thus, loss of CEACAM1 tumor suppressor gene expression in prostate cancer is due to aberrant chromatin acetylation. Results from this study will allow us to better understand the regulation of CEACAM1 gene during tumorigenesis and this may lead to design new therapy strategies to alter tumor progression or to implement early detection and prevention strategies.

14. SUBJECT TERMS

Prostate Cancer

15. NUMBER OF PAGES

52

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Title: Tumor-Specific Regulation of CEACAM1 Cell Adhesion Molecule in Prostate Carcinogenesis

Principal Investigator: Sue-Hwa Lin, Ph.D.

(4) INTRODUCTION

CEACAM1 (previously named C-CAM1) is a 105 kDa glycoprotein originally identified as a protein that mediates intercellular adhesion (1). Down-regulation of CEACAM1 was observed in many tumor types including prostate (2, 3), colon (4), endometrium (5), breast (6, 7), and hepatocellular (8) carcinomas suggesting that CEACAM1 may have an important role in the maintenance of normal epithelial phenotype. In experimental tumor models, reduction of CEACAM1 levels in normal rat prostate NbE cells promoted tumorigenesis (9), whereas re-expression of CEACAM1 in prostate cancer cells suppressed their tumorigenicity *in vivo* (9). Suppression of tumorigenicity by CEACAM1 was also observed in breast (10), bladder (11), and colon carcinoma (12). In addition, the human (13), rat (9), and mouse homologues of CEACAM1 (12) were all shown to have tumor-suppressive activity. These results support the role of CEACAM1 as a tumor suppressor.

The mechanism by which CEACAM1 is lost during tumorigenesis is not clear. Allelic loss of CEACAM1 gene, localized at chromosome 19 in humans (14) and 7 in mouse (15), has not been reported to occur in either prostate or colon cancer. Although an extensive analysis on human prostate cancer specimens has not been performed, studies by Rosenberg et al. (16) using tissues or cells from mouse colon carcinoma showed that neither chromosomal rearrangements nor gene deletions occurred close to the CEACAM1 gene. Thus, it is likely that down-regulation rather than irreversible loss of CEACAM1 expression is the major cause of tumorigenesis *in vivo*.

We propose to **identify mechanisms that regulate CEACAM1 gene expression in prostate carcinogenesis.** The specific aims for the phase I proposal are:

(1) Examine whether methylation of CEACAM gene is a mechanism for CEACAM down-regulation in prostatic carcinogenesis;

(2) Characterize transcription factors that regulate CEACAM expression in prostatic carcinogenesis.

The specific aims for the phase II proposal, which is continuation of phase I study, are:

(1) examine whether altered chromatin remodeling contributes to CEACAM1 down regulation;

(2) study interactions between known transcription factors and CEACAM1 promoter; specially examine the involvement of AP-2 in CEACAM1 gene expression in tumorigenesis;

(3) screen for factors that modulate CEACAM1 expression.

(5) BODY

The proposed work was divided into Tasks to be carried out in parallel.

Phase I Proposal

Task I-1. Examine whether cytidine methylation of regulatory sequences in CEACAM gene occurs in human prostatic carcinogenesis

Task I-2. Examine the involvement of AP-2 in CEACAM gene expression in carcinogenesis

Task I-3. Search for the transcriptional activators/co-activators involved in CEACAM expression

Task I-4. Search for transcriptional repressors.

Phase II Proposal is an extension of Phase I and the Statement of Work is as follows.

Task II-1. Determine whether altered chromatin remodeling contributes to CEACAM1 down regulation.

Task II-2 (continuation of Task I-2). Study interaction between known transcription factors and CEACAM1 promoter, specifically AP-2.

Task II-3 (continuation of Task I-3). Search for the transcriptional activators/co-activators involved in CEACAM1 expression.

Task II-4 (continuation of Task I-4). Search for transcriptional repressors that regulate CEACAM1 expression.

A reviewer for our proposed work suggested that we pursue regulation of CEACAM gene expression by steroid hormones. We agree with this reviewer that steroid hormones are important regulators of prostate growth and differentiation and, therefore, we have included androgen regulation in our study of transcriptional regulation of CEACAM gene. With the assistance of Dr. Guido Jenster, an expert in the field of gene regulation by steroid hormones and a collaborator in the proposed study, we have made significant progress in this effort and a manuscript on this aspect of study was published in Molecular and Cellular Endocrinology. We will briefly describe this part of the work first.

5.1. Studies on androgen regulation of CEACAM gene expression--(also see reprint attached)

5.1.1. Rationale

Because CEACAM is an epithelial cell adhesion molecule and androgen is known to promote differentiation of prostatic epithelia, it is likely that CEACAM expression in prostate is regulated by androgen. In our previous studies using castration-induced prostate involution together with administration of androgen or antiandrogen, we found that expression of CEACAM in rat ventral prostatic epithelia was repressed by androgen (17, 18). A similar regulatory pattern was observed in

seminal vesicle, but not in other organs (e.g. liver and kidney). These observations suggest that regulation of CEACAM expression by androgen is tissue-specific. However, it is not clear whether androgen regulation of CEACAM expression in rat ventral prostate is due to a direct effect on CEACAM gene expression or an indirect effect mediated by stromal-epithelial interaction. Therefore, we investigate whether androgen receptor has a direct or indirect effect on CEACAM promoter.

5.1.2. Experimental Plan

The effect of androgen and androgen receptor on CEACAM gene expression was studied by co-transfecting a reporter plasmid containing CEACAM promoter sequence and an expression plasmid containing androgen receptor. Mutation analysis was performed to locate the androgen response element located in the CEACAM promoter.

5.1.3. Results

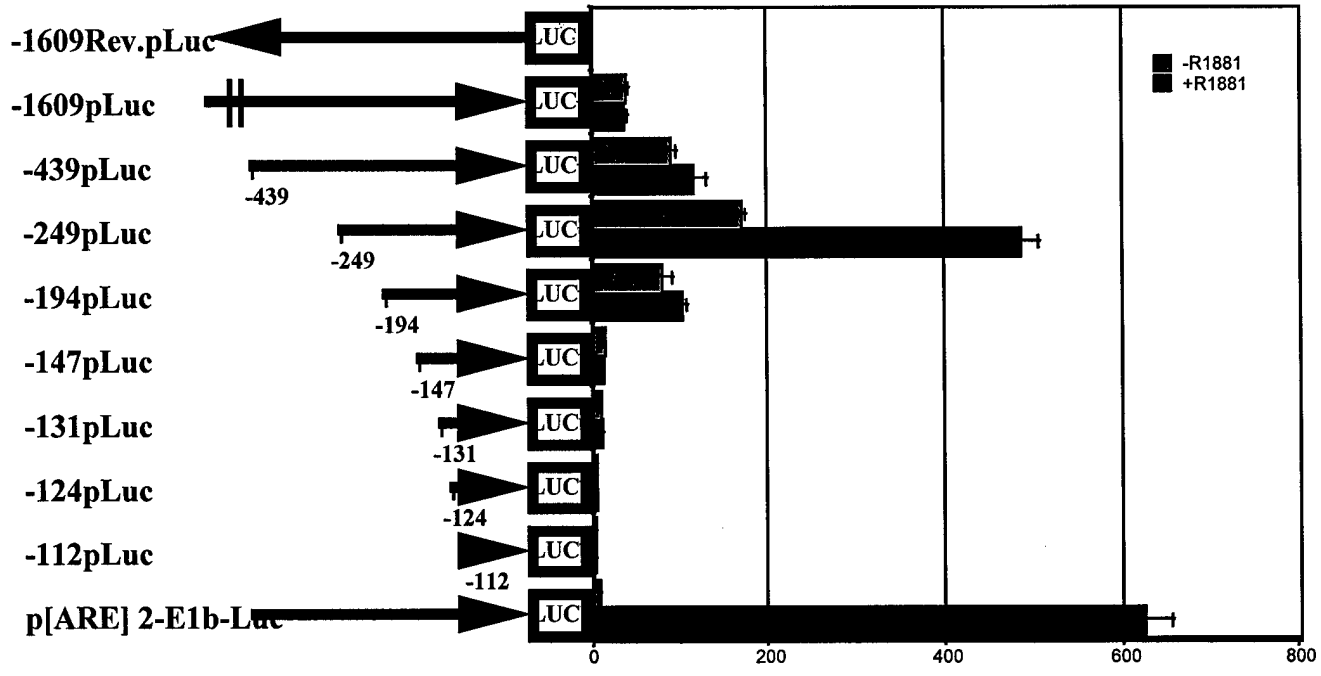
Localization of androgen-responsive region in CEACAM1 promoter

CEACAM promoters with differing length, constructed by 5' deletion, were cloned in front of luciferase gene in the reporter plasmid. Each of these plasmids, together with the androgen receptor expression vector pAR0, was transiently transfected into HeLa cells. The 1609 bp CEACAM promoter mediated a 106-fold increase in reporter gene (luciferase) expression without addition of androgen analogue R1881 (Fig. 1). Deletion of CEACAM promoter up to bp -194 did not have any significant effect on its ability to induce luciferase expression, while deletion up to bp -147 markedly reduced the promoter activity (Fig. 1). This result suggests that a minimal promoter is located within the first 194 bp 5' from CEACAM's translation start site.

We next investigate whether there is any androgen responsive sequence in the CEACAM 5' promoter region. As shown in Fig. 1, the plasmid containing CEACAM promoter region from -249 bp to -21 bp exhibited a two-fold increase in luciferase activity upon addition of androgen analogue R1881. Plasmid containing the 439 bp segment proximal to the translation start site also gave a moderate hormone response (1.5 fold). In contrast, no hormone response was observed with plasmids containing the entire 1609 bp or the 194 bp segment proximal to the translation start site. These observations suggest that the region between -249 and -194 bp in CEACAM promoter may contain an androgen-regulated sequence.

Fig. 1. Regulation of the CEACAM expression by androgen. A series of reporter plasmids containing CEACAM promoter fragments with different 5' deletions were co-transfected with wild-type androgen receptor plasmid (pAR0) into HeLa cells. Twenty hours post-transfection, cells were incubated with (+) or without (-) 1 nM R1881. Luciferase activities of these cell lysates were determined and reported

as averages ± S.D. in relative light units from triplicate transfections.

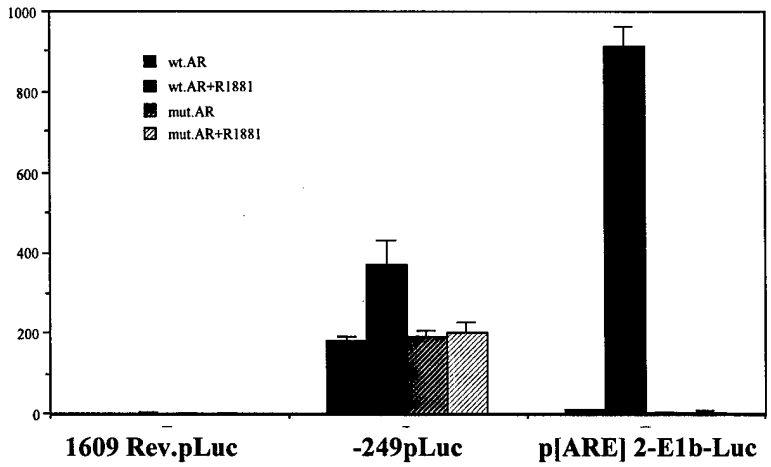


***In Vitro* Study**

Test the effect of mutant AR on CEACAM promoter activity

AR is a 100 kDa protein containing a DNA binding domain in its N-terminal region and a transcription activation domain in its C-terminal region. To test whether activation of CEACAM promoter by androgen is due to direct interaction between AR and CEACAM promoter, we have investigated the effect of mutant AR (AR64), which has a mutated (defective) DNA-binding domain, on CEACAM promoter activity. In contrast to the wild-type AR, this mutant AR (AR64), when co-transfected with p-249Luc into HeLa cells, did not show any detectable hormone induction (Fig. 2). This result suggests that activation of CEACAM promoter by wild-type AR is likely mediated by direct binding of AR to CEACAM promoter.

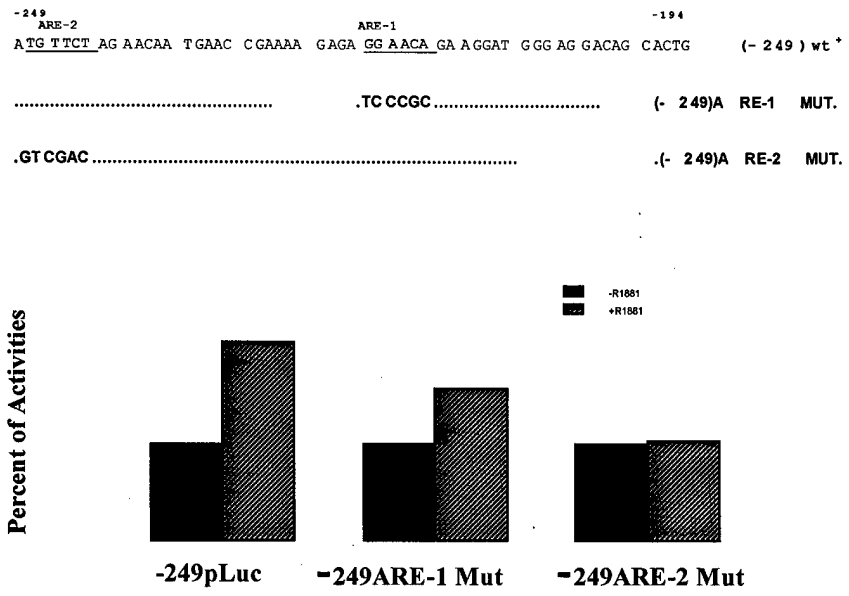
Fig. 2. Effect of AR mutant on its ability to activate CEACAM promoter. Cells were transfected with the -249Luc CEACAM promoter together with wild-type AR (pAR0) or mutant AR (pAR64) plasmid, respectively. Activities are presented as averages ± SD of triplicate transfections.



Identification of AR-interacting sites in CEACAM1 promoter

The consensus DNA-binding site for androgen receptor is GGA/TACANNNTGTTCT. Two potential androgen-responsive element (ARE) half sites, GGAACA and TGTTCT, located at bp -215 and -248 in CEACAM 5' promoter region, respectively, were found (Fig. 3). These half sites are found in the same region, i.e., between not -249 and -197 in CEACAM 5' promoter, where the androgen-regulated sequence was identified. These two potential ARE half sites (ARE-1 and ARE-2) were mutated to see if they are indeed involved in androgen regulation. Mutation of the first site from GGAACA to TCCCGC (ARE mutant #1) did not have any detectable effect on CEACAM promoter's response to R1881, suggesting that this sequence is probably not involved in androgen regulation of CEACAM. On the other hand, mutation of the second site from TGTTCT to GTCGAC abolished the androgen-stimulated response. These results suggest that the sequence between no -248 and -243 (ARE-2) is critical for androgen regulation of CEACAM, whereas that between not -215 and -210 (ARE#1) is not.

Fig. 3. Mutational analysis of the two putative androgen-responsive elements. Cells were transfected with reporter plasmids containing the -249 bp promoters whose putative ARE-1 or ARE-2 sequence was mutated. Activities are presented as percent of that of the corresponding plasmid containing no mutation and without R1881 treatment.



5.1.4. Discussion

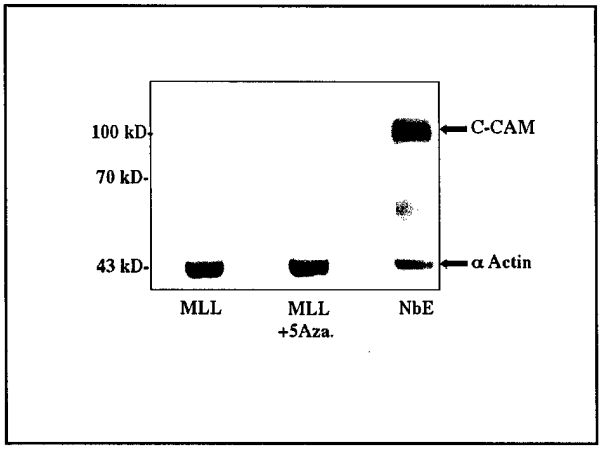
We show that AR up-regulates CEACAM transcription in a ligand-dependent manner and this up-regulation requires a short (-249) promoter. In addition, AR, through its DNA binding domain, directly interacts with CEACAM promoter. These observations suggest that AR regulation of CEACAM expression is, at least in part, mediated by a direct mechanism. This study also establishes that androgen receptor is one of the transcriptional regulator of CEACAM gene.

5.2. Studies performed under Task I. 1-- Examine whether cytidine methylation of regulatory sequences in CEACAM gene occurs in human prostatic carcinogenesis

Aberrant hypermethylation of 5' CpG islands within proximal promoter regions has been implicated as a mechanism by which tumor suppressor genes can be inactivated. Examples of this mechanism have been best demonstrated for the VHL and p16 tumor suppressor genes (19, 20). In addition, Graff et al. (21) have also shown that hypermethylation is one of the mechanisms for the down-regulation of the "metastasis suppressor" gene, i.e., E-cadherin, in prostate cancer. Genomic clone containing the CEACAM1 promoter has been isolated (22). Promoter sequence analysis showed that CEACAM1 promoter does not have high CpG content typical for genes whose expressions were modulated by methylation. Only 5 CpG dinucleotides were found within the 250 bp promoter proximal region of CEACAM1 promoter. Treatment of the Mat-LyLu cells with DNA methylation inhibitor 5-aza-2'-deoxycytidine did not result in an increase of CEACAM1 protein

expression as judged by RT-PCR and Western blot analysis (Fig. 4). Thus, promoter methylation was unlikely to be involved in CEACAM1 regulation. This observation is also consistent with the report by Rosenberg et al. (16) that methylation was not detected in mouse CEACAM1 gene in colon carcinoma samples examined.

Fig. 4. Effect of 5-deoxyazacytidine on CEACAM expression. Mat-Ly-Lu (MLL) cells (1 x 10^6 cells) were treated with (+) or without (-) 1 uM of 5-deoxyazacytidine for 3 days. The cells were lysed in SDS sample buffer and analyzed by western immunoblot using antibody against CEACAM. NbE cells, which is a normal prostate epithelial cell line derived from Noble rat, was used as a positive control.



5.3. Studies performed under Task I. 2 (also Task II.2) --Examine the involvement of AP-2 in CEACAM gene expression

5.3.1. Rationale

The CEACAM promoter does not have TATA or CAAT box but has potential binding sites for known basal and regulatory transcriptional factors. Several AP-2 (activator protein 2) binding sites were found in the CEACAM promoter. AP-2 was shown to regulate the expression of several oncogenes and tumor suppressor genes and has been shown to involve in tumorigenesis (23, 24). Thus, AP-2 is one of the known factors that may be potentially involved in CEACAM down-regulation in tumorigenesis.

5.3.2. Experimental Plan

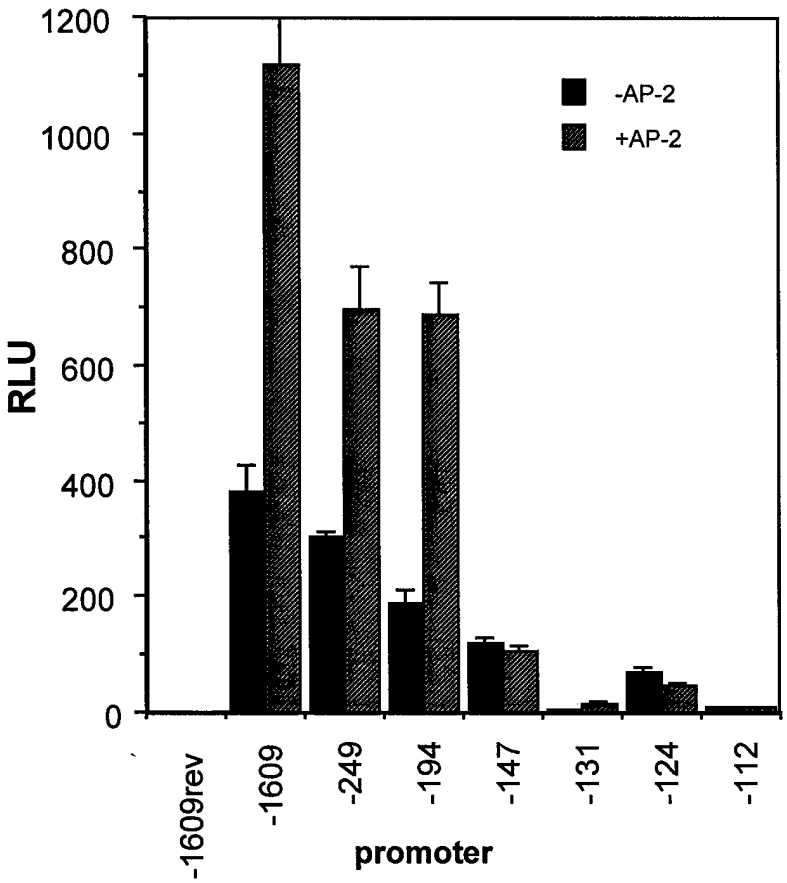
We examine whether AP-2 has any effect on CEACAM promoter activity by co-transfection CEACAM promoter plasmid constructs containing various length of CEACAM promoter and an AP-2 expression vector into Mat-Ly-Lu cells.

5.3.3. Results

CEACAM promoters with differing length, constructed by 5' deletion, were cloned in front of

luciferase gene in the reporter plasmid. Each of these plasmids was transiently co-transfected with or without the mammalian expression plasmid containing AP-2 gene into HeLa cells. Addition of AP-2 resulted in a 2.5-fold increase in reporter gene (luciferase) expression when the reporter gene was driven by CEACAM promoter containing 1609, 249, or 194 bp (Fig. 5). Deletion of CEACAM promoter down to 147 bp abolished the AP-2 effect on CEACAM promoter (Fig. 5). This result suggests that AP-2 is one of the transcriptional activator of CEACAM promoter and the AP-2 responsive element is located between -194 bp to -147 bp region of the CEACAM promoter. This observation is consistent with the AP-2 binding site predicted from sequence analysis.

Fig. 5. Regulation of the CEACAM expression by AP-2. A series of reporter plasmids containing CEACAM promoter fragments with different 5' deletions were co-transfected with mammalian expression vector containing AP-2 gene into HeLa cells. Luciferase activities of these cell lysates were determined and reported as averages ± S.D. in relative light units from triplicate transfections.



AP-2 does not bind to CEACAM promoter directly

This result suggests that AP-2 is a transcriptional activator of CEACAM and thus is a potential regulator of CEACAM expression during tumorigenesis. To further examine whether AP-2 binds to CEACAM promoter, EMSA was performed using double strand oligonucleotide containing –147 to –194 sequence of CEACAM promoter. We found that AP-2 did not bind to CEACAM promoter in EMSA (data not shown), suggesting that AP-2 modulates CEACAM promoter activity indirectly through other transcription factors.

5.3.4. Discussion

These results suggest that AP-2 is a transcriptional activator of CEACAM1 and thus is a potential regulator of CEACAM1 expression during tumorigenesis. Recently, Ruiz et al. (23) showed that AP-2 expression is associated with luminal differentiation and is lost in prostate cancer. This pattern of expression of AP-2 in prostate and during prostate cancer progression is very similar to those of CEACAM1 (3, 25). In addition, it was also shown that AP-2 inhibited tumorigenicity and represses vascular endothelial growth factor transcription in prostate cancer cells (24). Our studies suggest that activation of CEACAM1 transcription is one of mechanisms involved in the tumor suppressive effect of AP-2.

5.4. Studies performed under Task I-3 (also Task II-3) --Search for the transcriptional activators/co-activators involved in CEACAM expression

5.4.1 Rationale

We hypothesized that decrease in CEACAM transcription during tumorigenesis may be due to loss of activator(s) or coactivator(s). Such a factor should have the following properties: It should (1) exhibit different levels of expression between normal and cancer cells; (2) be able to activate CEACAM expression in CEACAM negative cells; and (3) suppress tumorigenicity of prostate cancer cells. We will identify the potential tumor-specific transcription factors based on these functional criteria.

5.4.2. Experimental plan

We plan to functionally identify these factors in the context of native CEACAM promoter. In brief, the CEACAM promoter will be constructed in front of a green fluorescence protein (GFP) reporter gene. Activation of CEACAM promoter by transcription factors will stimulate the expression of GFP. Cells with elevated level of GFP can be selected by fluorescence-activated cell sorting (FACS) analysis. Plasmids in GFP-positive cells will be isolated for further analysis.

5.4.3. Results

Construction of CEACAM promoter into pEGFP-1 promoter-reporter vector to generate plasmid pCAM-GFP (as proposed)

The 1609 bp CEACAM promoter was isolated from plasmid pGL-1609 by XhoI and HindIII restriction-enzyme digestion. This 1609 bp promoter sequence was then cloned in front of green fluorescence protein gene in the reporter plasmid, pEGFP-1 (Clontech, CA).

Transfect pCAM-GFP into PC3 cells and select for transfectants by G418 to establish PCEACAM-GFP cells (as proposed)

The plasmid pCAM-GFP was transfected into PC3 cells with the aid of DOTAP (Behringer Menheim Biochemical) and the cells that contained the pCAM-GFP plasmid were selected by G418. Several clones (PC-CAM-GFP) were selected and expanded for further study.

FACS and western blot analysis of PCEACAM-GFP cells (as proposed)

The CEACAM promoter activity in PC-3 cells was measured by FACS and western blot analysis. Six PC-3 cell lines (PC-CAM-GFP-1, PC-CAM-GFP-2, PC-CAM-GFP-3, PC-CAM-GFP-4, PC-CAM-GFP-5 and PC-CAM-GFP-6) were tested and the parental PC-3 cells were used as a control. Both FACS analysis and western immunoblot using polyclonal antibody against GFP were employed to measure the amount of GFP protein produced. The PC-3 control cells did not have GFP expression, while transfection of PC-3 cells with the PCEACAM-GFP construct produced 10- to 100-fold increases in fluorescence intensities, suggesting that GFP was produced from the pCAM-GFP construct (data not shown). Western immunoblot analysis showed that significant amounts of GFP protein were detected in the PC-CAM-GFP cell lines (data not shown). This observation suggests that the increases in fluorescence intensities in PC-CAM-GFP cells indeed resulted from the production of GFP rather than from non-specific auto-fluorescence of the transfected cells.

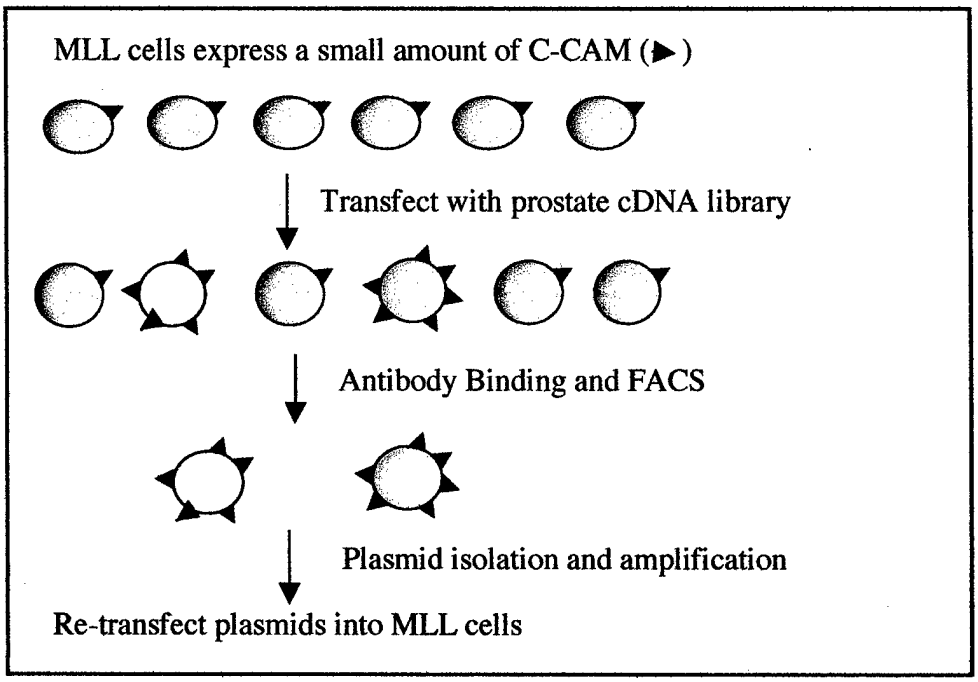
Interpretation of PC-CAM-GFP FACS analysis results

Although CEACAM is not expressed in PC-3 cells, we have observed that the 1609 bp CEACAM promoter was able to activate GFP expression in PC-3 cells. This observation suggests that either the 1609 bp CEACAM promoter region does not contain the tumor-regulated element, or this 1609 bp promoter fragment is not sufficient to elicit the tumor-specific regulatory event, or the insertion of GFP reporter plasmid into PC-3 cells causes a constitutive activation of GFP.

Rationale for selecting Mat-LyLu rat prostate cancer cell line for further study (modification based on the negative results obtained)

Based on the result shown above, it is obvious that a longer promoter would be needed to study the tumor-specific transcriptional regulatory event. Alternatively, the study can be performed in a tumor cell line in which the level of CEACAM expression was down-regulated and modulation of CEACAM expression can be easily detected. The latter approach has the advantage of not being limited by the size of promoter obtained and also has the advantage of studying CEACAM regulation in its natural environment. Since CEACAM is a membrane protein, cells that have increased CEACAM expression can be selected by CEACAM antibody binding followed with fluorescence activated cell sorting (FACS). The experimental scheme for this approach is shown in Fig. 6.

Fig. 6. Functional screening of molecules that activate CEACAM expression

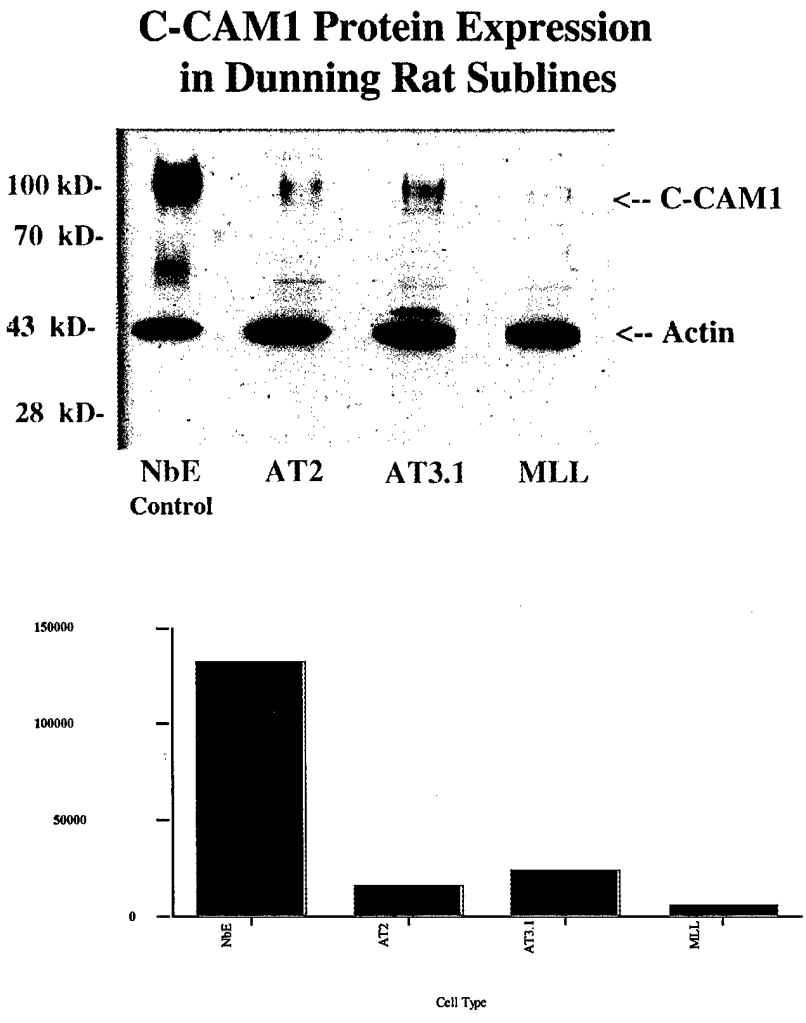


We found that Dunning rat prostate cancer cell lines have the property that is suitable for our purpose. The Dunning 3327 prostate cell line was isolated from a rat prostate tumor by Dunning (26) from a 22-month-old inbred Copenhagen male rat. Following serial *in vivo* passage of the original R3327 tumor, sublines with different biological characteristics were obtained and characterized (27). These cell lines represent tumors ranging from relatively benign, slowly growing, differentiated, androgen-sensitive tumors to rapidly growing, anaplastic, hormone-insensitive malignant tumors. If the level of CEACAM protein expression in this series of cell lines showed distinct tumor-specific down-regulation, these cell lines will be suitable for studying tumor-specific regulation of CEACAM expression. Therefore, we first characterized CEACAM expression in this series of Dunning cell lines.

Western immunoblot analysis of CEACAM gene expression in the Dunning series prostate cancer cell lines

The levels of CEACAM protein expression in these cell lines were examined by western immunoblot analysis. A normal prostate cell line NbE, derived from ventral prostate of Noble rat (28), was used as a control. As shown in Fig. 7, distinct changes occurred in CEACAM expression in the Dunning prostate cancer cell lines. A significant decrease in CEACAM protein levels occurred at the transition from normal to carcinoma, i.e. Dunning 3327, followed with further reduction in CEACAM protein level in the rapid growing tumors, i.e. AT-3 and Mat-LyLu. In Mat-LyLu cell line, CEACAM expression level is about 5% as compared to that of normal control cell line. Due to this low CEACAM level, we chose to use Mat-LyLu cell line for further studies.

Fig. 7. Western immunoblot analysis of CEACAM expression in Dunning rat prostate cancer cell lines.



Transfection of human prostate cDNA library in mammalian expression vector into Mat-LyLu and FACS analysis

To screen for CEACAM activation factors present in normal prostate, a human prostate cDNA library in mammalian expression vectors was constructed and used to transfect Mat-LyLu cells. Three million Mat-LyLu cells were transfected with 15 ug of expression vector using lipofectin (Gibco/BRL) according to manufacturer's instruction. At 2 days post-transfection, these cells were trypsinized from the plates and incubated with polyclonal anti-CEACAM antibodies followed with FITC-conjugated secondary antibody. The top 10% fluorescence positive cells, which were considered CEACAM positive, were separated from total cell populations by FACS.

Isolation of plasmid DNA from CEACAM positive cells

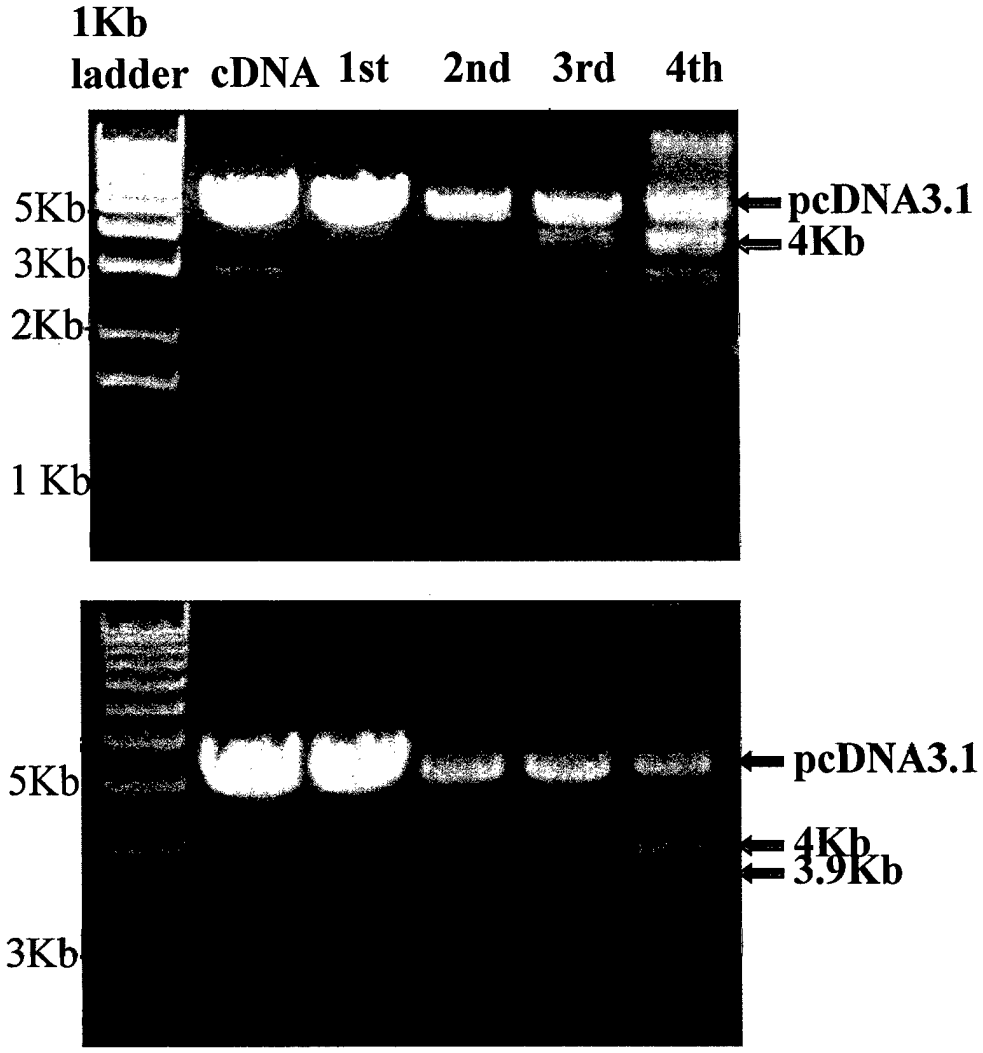
The selected cells were concentrated by centrifugation and the DNA was isolated with phenol/chloroform extraction followed with ethanol precipitation. Plasmid DNA, which contained b-lactamase gene, can confer ampicilin resistance and was retrieved from the total DNA by electroporation of total DNA into E. coli. Transformed bacteria were selected in ampicilin-containing agar plates. Fifty thousand ampicilin resistance colonies were recovered from the first round of selection. These bacteria colonies were combined and their plasmid DNA (first round DNA) were prepared using a Maxi-prep kit from Qiagen.

The first round DNA (15 ug) were used to transfect seven millions of Mat-LyLu cells. The highest 4.5% CEACAM positive cells were FACS sorted and the plasmid DNA (second round DNA) were retrieved as described above. The same procedures were repeated two more times and the resulting DNA were analyzed by restriction enzyme digestion.

Results from 4 cycles of FACS selection

Restriction digestion of plasmids after four-cycle sorting is shown in Fig. 8. The original cDNA library contained a series of DNA inserts with various sizes and appeared on the gel as a smear pattern. Enrichment of certain insert sizes was observed following four cycles of selection (Fig. 8). The plasmids from the fourth selection were transformed into bacteria and plasmid from single bacteria colony was isolated. The DNA sequences of these plasmid inserts were determined by DNA sequencing analysis. Five of the plasmids gave readable DNA sequences. One of the plasmid encodes histone deacetylase 1, two encode zinc finger protein (kid-1), and two of them do not have match with gene bank data base.

Fig. 8. Restriction digest profile of the plasmids isolated from cells selected from FACS sorting. The lower panel is the longer electrophoresis run of first panel.



5.4.4. Discussion

Our effort on the search for activator/co-activators that regulate CEACAM gene expression during tumorigenesis has yielded significant insights. We found that GFP is not a suitable reporter for promoter study. This is illustrated by our first approach using a CEACAM promoter-driven GFP construct. Although CEACAM is not expressed in PC-3 cells, the 1609 bp CEACAM promoter had basal promoter activity and activate GFP expression in PC-3 cells. We thus used an alternative approach by using a tumor cell line in which the level of CEACAM was down-regulated. We have found that Mat-LyLu prostate cancer cell line is suitable for our purpose. We have obtained some interesting results using this cell lines and will continue this line of investigation.

The concept of functional screening of molecules that modulate CEACAM1 expression in tumor cells was considered to be novel, but risky, by the reviewers. With the funding, we were able to evaluate the method and improve the efficiency and accuracy of the steps taken. Our modifications include (1) Use of the rat MLL cells instead of human prostate cancer cell lines. The reason is that the CEACAM1 antibodies react with rat CEACAM well but only react weakly with human CEACAM1. This cell line also allows us to study CEACAM1 gene regulation in its natural context. (2) We have compared several transfection methods/reagents and found that lipofectamine can produce about 30% transfection efficiency in MLL cells. (3) Electroporation of isolated plasmids into competent E. coli greatly increases the efficiency of transformation. This step not only amplifies selected plasmids, but also removes mitochondria or cellular DNA that are present in the DNA preparation. With these improvements, we are confident that factors that activate CEACAM1 expression can be isolated.

5.5. Studies performed under Task I-4 (also II-4)— Search for transcriptional repressors

5.5.1. Rationale: Down-regulation of CEACAM1 expression during prostate cancer progression may also arise from increase of factors that suppress CEACAM1 expression.

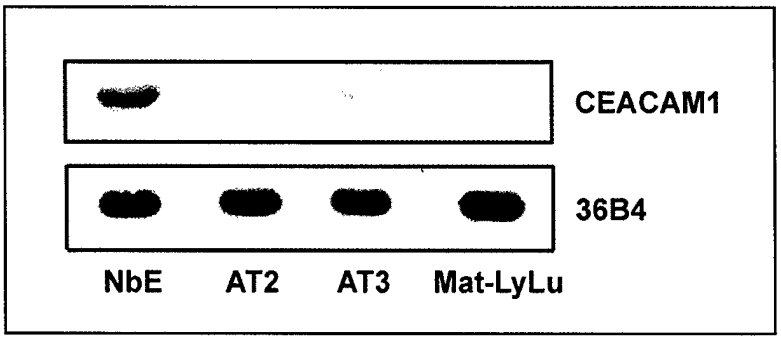
5.5.2. Experimental Plan: CEACAM1 promoter regions that show different activity when expressed in normal versus tumor cell lines will be used to probe for the transcription factor that bind to that region differentially.

5.5.3. Results

CEACAM1 Messages in Prostate Cancer Cell Lines

As shown in Fig. 7, CEACAM protein was down-regulated in Mat-LyLu cell line. Since decrease in protein expression can arise from transcriptional and post-transcriptional events, we further examine the levels of CEACAM1 messages in the NbE and Mat-LyLu cell lines. Using a probe generated from full-length CEACAM1 cDNA, Northern blotting showed a significant decrease in the steady-state levels of 4 kilobase (kb) mRNA for CEACAM1 in Mat-LyLu cells (Fig. 9). These observations indicate that loss of CEACAM1 protein is due to the reduction of CEACAM1 transcript. Thus, down-regulation of CEACAM1 expression in prostatic cancer cells could be due to altered transcriptional activity.

Fig. 9. Down-regulation of CEACAM1 message in prostate cancer cells. Northern blot analysis of CEACAM1 expression. The levels of CEACAM1 messages in NbE, AT2, AT3, and Mat-LyLu cells were tested by Northern blot analysis using a probe generated from the full-length CEACAM1 cDNA (29).

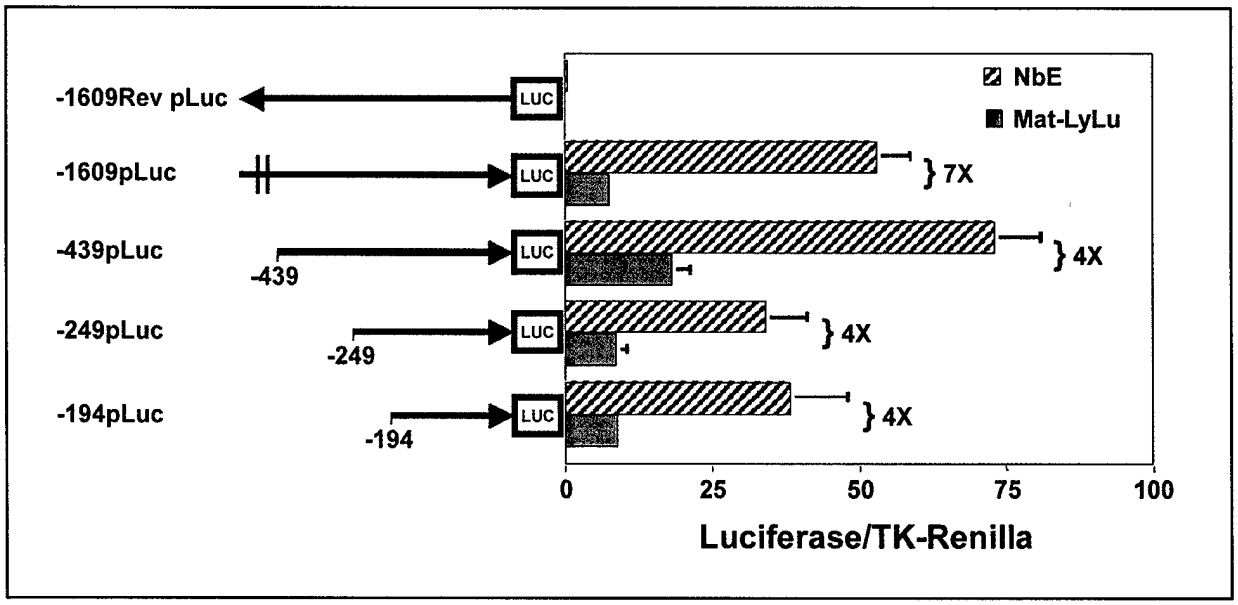


Transcriptional regulation of CEACAM1 gene expression in prostatic cancer cell lines

We next examined how transcriptional regulation of CEACAM1 gene occurs in tumorigenesis. Previous deletion analysis identified a minimal promoter located between nt -194 and -147 proximal to the CEACAM1 translation start site (22, 30). To compare promoter activities in the CEACAM1 positive and negative cells, we transfected the CEACAM1 promoter-luciferase constructs into normal NbE and Mat-LyLu prostatic cancer cells. A similar pattern of promoter activity was observed in the -1609 bp, -439 bp, -249 bp and -194 bp CEACAM1 promoter reporter constructs in the Mat-LyLu cells as compared with the NbE cells (Fig. 10). However, there is a reproducible 4-fold decrease in the overall CEACAM1 promoter activity in the fast growing malignant Mat-LyLu cells. The –147 bp CEACAM1 promoter was inactive in either cell types (data not shown). These results suggest that down-regulation of CEACAM1 in prostate cancer cells is correlated with altered CEACAM1 promoter activity and that a tumor-specific promoter regulatory activity lies between nt -194 and -147 in the minimal CEACAM1 promoter.

Fig. 10. Comparison of CEACAM1 promoter activities in NbE and Mat-LyLu cells. A series of reporter plasmids containing various lengths of the CEACAM1 promoter were transfected into NbE or Mat-LyLu cells as described in Materials and Methods. CEACAM1 promoter (-1609 bp) cloned in reverse orientation (-1609Rev pLuc) was used as a reference. NbE or Mat-LyLu cells transfected with pTK-Renilla were used to normalize the transfection efficiency. After normalization by Renilla luciferase activities, the average luciferase activities ± S.D. of triplicate transfections were shown. Values located at the right of each construct indicate the fold difference in the promoter activity in NbE cells compared with that observed for Mat-LyLu cells. The experiment was repeated three times with triplicate transfections for each construct and similar results were obtained. Results from one of the

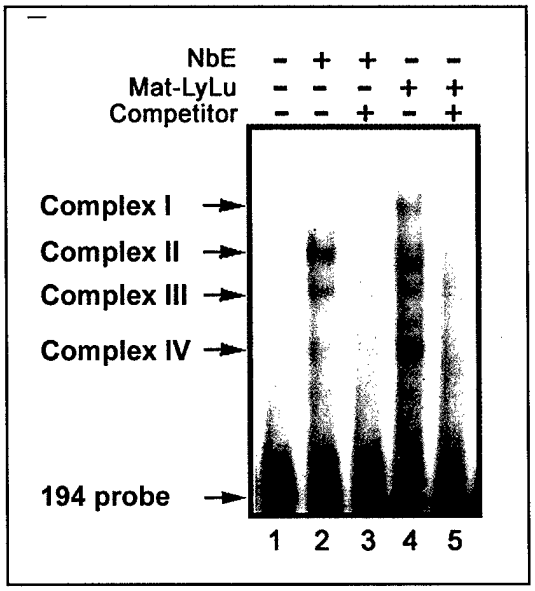
experiments are shown.



Binding of NbE and Mat-LyLu Nuclear Extracts to CEACAM1 Promoter

To identify the tumor-specific activity at the CEACAM1 promoter, we employed gel shift assays (EMSA) to examine binding of nuclear proteins from NbE versus Mat-LyLu cells to a double-stranded oligonucleotide containing the minimum CEACAM1 promoter (nt -194 to -147, 194probe). Four major bands (complex I to IV) were detected in the Mat-LyLu (Fig. 11, *lane 4*) nuclear extract. The binding of complex I to IV is specific as it can be competed by an excess of unlabeled 194probe (Fig. 11, *lane 5*). In contrast, three major bands (complex II to IV), which can be competed by an excess of unlabeled 194probe, were detected in the NbE nuclear extract (Fig. 11, *lane 2 versus 3*). As complex I is only present in the Mat-LyLu extract, we investigated the possibility that the protein in this complex may play a role in the decreased CEACAM1 promoter activity in Mat-LyLu cells.

Fig. 11. EMSA of the interaction between NbE or Mat-LyLu nuclear extract with the 194probe. Oligonucleotides corresponding to the region between -194 to -147 bp of the CEACAM1 promoter were synthesized and used as probe (194probe). Nuclear extracts from NbE or Mat-LyLu cells were used. Positions of shifted complexes (complexes I to IV) were indicated by arrows.

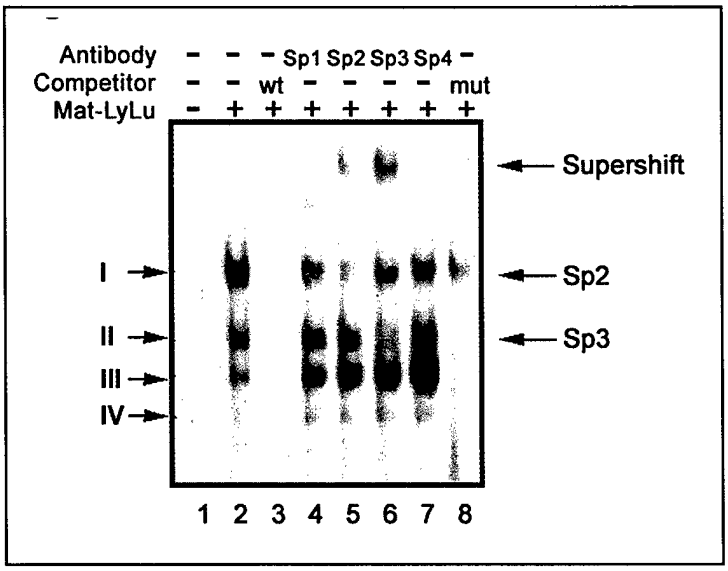


Involvement of Sp Transcription Factor Family at the CEACAM1 Promoter

Sequence analysis showed that the 194 bp region of the CEACAM1 promoter is highly GC-rich and contains elements that match the consensus binding sequence for Sp1 (22). To determine if Sp family of transcription factors are present at the CEACAM1 promoter, we used antibodies against Sp1, Sp2, Sp3, and Sp4 to identify the protein(s) present in complex I in Mat-LyLu cells. Complex I was shifted by anti-Sp2 antibody, as shown by the decrease in complex I intensity (Fig. 12, *lane 5*). Complex II was shifted by anti-Sp3 antibody (Fig. 12, *lane 6*). Anti-Sp1 antibody generated a weak supershifted band with no significant decrease in the intensity of any of the complexes, suggesting that Sp1 is a minor component in these complexes (Fig. 12, *lane 4*). Anti-Sp4 antibody did not generate any supershifted band (Fig. 12, *lane 7*). Because Sp2 is present in complex I, it is likely that Sp2 is one of the transcription factors that suppresses CEACAM1 promoter activity in Mat-LyLu cells.

Fig. 12. EMSA in the presence of Sp1, Sp2, Sp3, and Sp4 antibodies. Gel shift analysis using the 194probe was performed in the absence or presence of antibodies against Sp1, Sp2, Sp3, or Sp4 (*Lanes 4 – 7*). *Lane 4*, anti-Sp1 antibody generated a weak supershifted band, however, no significant decrease was observed in any of the complexes, suggesting Sp1 might be a minor component in these complexes. *Lane 5*, complex I was shifted by anti-Sp2 antibody as judged by the decrease in complex I intensity. *Lane 6*, complex II was shifted by anti-Sp3 antibody. *Lane 7*, none of the

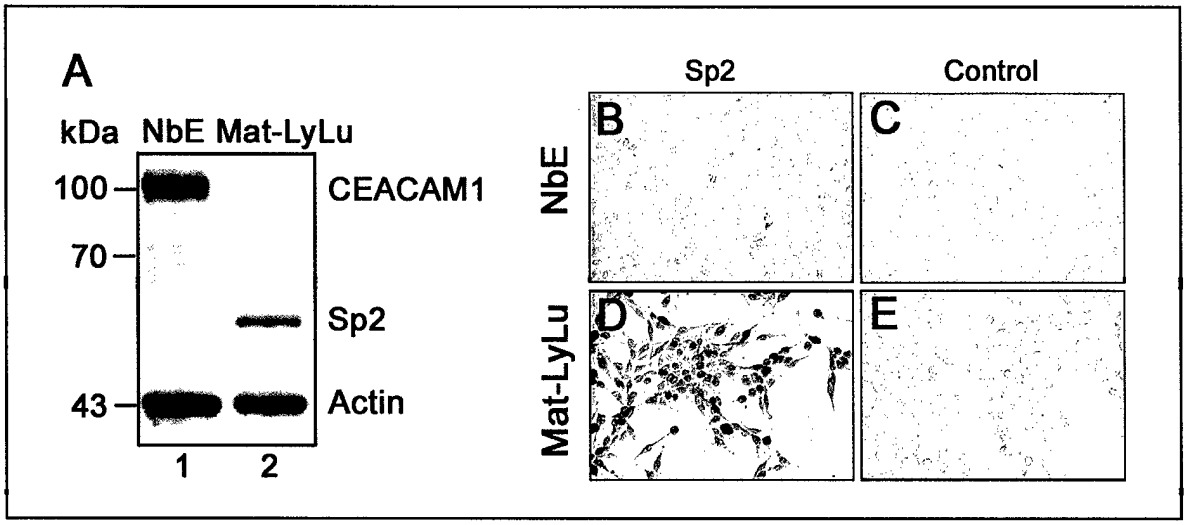
complexes was shifted by anti-Sp4 antibody. *Lane 8*, complex I was not competed by the presence of 100-fold molar excess of the 194mut probe.



Levels of Sp2 Protein in Cells Correlate with CEACAM1 Down-regulation

Down-regulation of CEACAM1 in Mat-LyLu cells might be due to an increase in the concentrations of Sp2 in Mat-LyLu cells in comparison to NbE cells. Western blot analysis showed that the Sp2 level in Mat-LyLu cells was 10-fold higher than that in NbE cells, and is inversely correlated with CEACAM1 levels in these cells (Fig. 13*A*). Further, staining of Sp2 protein was higher in Mat-LyLu than NbE cells by both immunofluorescence (not shown) and immunoperoxidase detection (Fig. 13, *B – E*). Consistent with the role of Sp2 as a transcription factor, immuno-localization analysis revealed stronger nuclear than cytoplasmic staining of Sp2 in Mat-LyLu cells (Fig. 13*D*). Higher levels of Sp2 in Mat-LyLu than NbE cells suggest a potential repressive function of Sp2 on CEACAM1 gene expression in prostate cancer cells.

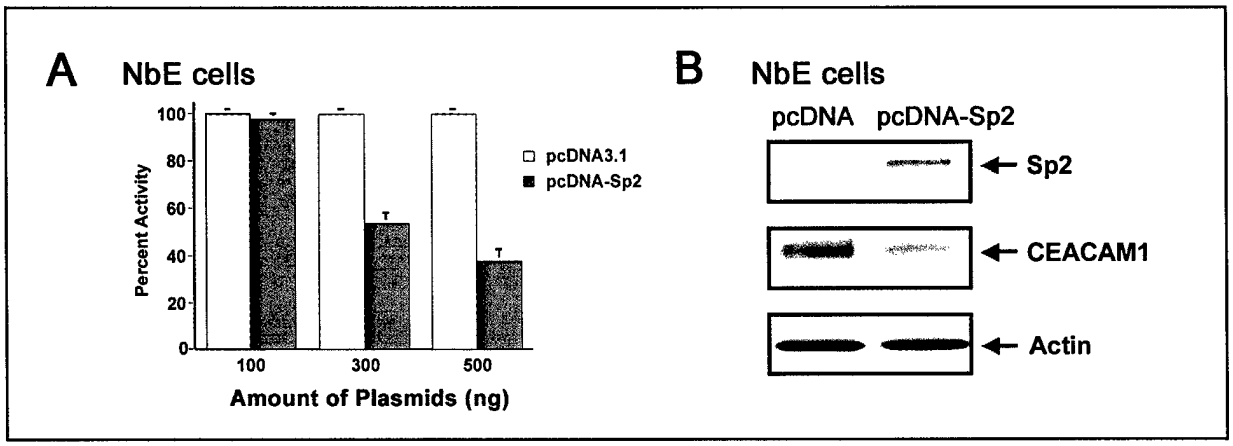
Fig. 13. Sp2 expression correlates with CEACAM1 down-regulation. *A*, Differential expression of Sp2 in NbE versus Mat-LyLu cells. Cell lysates (20 μg) prepared from NbE or Mat-LyLu cells were boiled in SDS sample buffer and the proteins were separated by SDS-PAGE. The proteins were transferred onto nitrocellulose membrane and blotted sequentially with anti-Sp2, anti-CEACAM1 and anti-actin antibodies. *B*, Localization of Sp2 in NbE cells. *D*, Localization of Sp2 in Mat-LyLu cells. Mat-LyLu or NbE cells grown on coverslips were fixed by formaldehyde and immunostained with anti-Sp2 antibody. The localization of Sp2 protein was detected by diaminobenzidine. NbE (*C*) or Mat-LyLu (*E*) cells stained with secondary antibody alone were used as controls.



Suppression of CEACAM1 Gene Expression by Sp2

To investigate whether Sp2 has a direct suppressive effect on the CEACAM1 promoter, we transfected NbE cells, which have low to undetectable levels of Sp2, with Sp2 expression vector and CEACAM1 promoter reporter construct. Overexpression of Sp2 resulted in a dose-dependent inhibition of CEACAM1 promoter activity as compared to an empty pcDNA3.1 vector control (Fig. 14*A*). This Sp2-mediated suppression was also observed on endogenous CEACAM1 protein levels in Sp2 transfected NbE cells (Fig. 14*B*). These results suggest that Sp2 is one of the factors that mediate a decrease in CEACAM1 expression in Mat-LyLu cells. Taken together, these results are consistent with the interpretation that Sp2 suppresses the expression of CEACAM1 in prostate cancer cells.

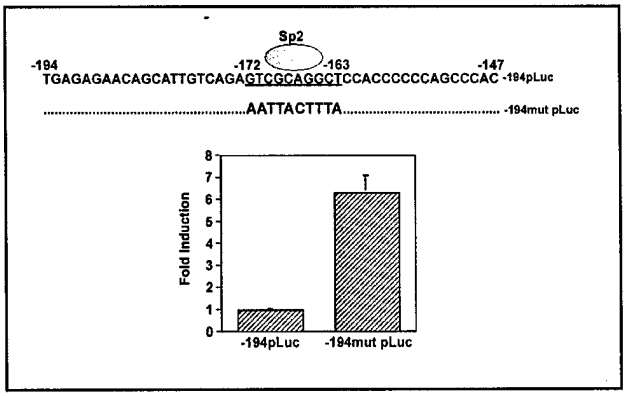
Fig. 14. Suppression of CEACAM1 expression by Sp2. *A*, Sp2 decreases transcriptional activity of CEACAM1 promoter in NbE cells. NbE cells were co-transfected with CEACAM1 promoter reporter plasmid (-1609pLuc) and various amounts of pcDNA-Sp2 expression vector or control vector pcDNA3.1 as indicated. The luciferase activity is presented as a percentage of that of control plasmid transfected cells. The data are presented as the mean ± S.E. of three independent experiments. *B*, Increased expression of Sp2 inhibited endogenous CEACAM1 expression in NbE cells. NbE cells were transfected with control expression vector pcDNA3.1 or pcDNA-Sp2 expression vector. Twenty four hours after transfection, the cells were harvested and lysed in RIPA buffer. Equal amounts of proteins were loaded and electrophoresed on a 4 – 12% SDS-polyacrylamide gel. Western immunoblot analysis was performed by using anti-Sp2 antibody and anti-CEACAM1 antibody (Ab669). The expression of actin was used as a control.



Sp2 binding at the CEACAM1 Promoter

Sp2 is known to bind to GT-box sequences (31, 32) and a GT-box like sequence is located between nt -172 to –163 at the CEACAM1 promoter. We mutated the GT-box sequences to generate a 194-mut oligo and used it as a competitor in EMSA. Addition of 100-fold excess of the 194-mut oligo blocked complex II-IV formation but did not alter complex I formation (Fig. 12, *lane 8*). This suggests that Sp2 binds to the GT-rich sequence between nt-172 to -163 in the CEACAM1 promoter. We next generated a -194pLuc reporter containing the Sp2 site mutation (-194mutpLuc) and determined whether the loss of Sp2 binding results in an increase in CEACAM1 promoter activity in Mat-LyLu cells. Mutation of the Sp2 binding site reproducibly resulted in a 6 to 7-fold increase in CEACAM1 promoter activity in Mat-LyLu cells when compared to that of the wild-type CEACAM1 promoter (Fig. 15). This suggests that binding of Sp2 to nt -172 to –163 of the CEACAM1 promoter suppresses CEACAM1 promoter activity in prostate cancer cells.

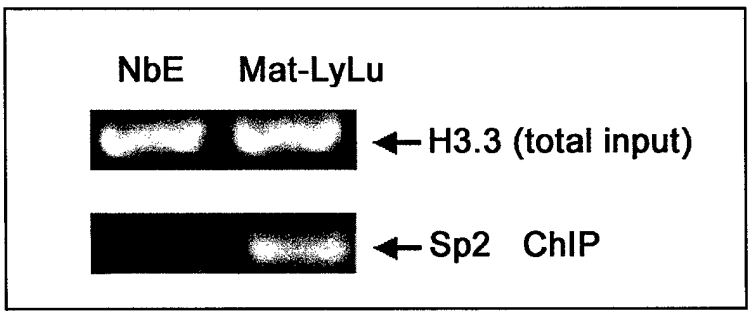
Fig. 15. Mutation of Sp2 binding site increases CEACAM1 promoter activity. Mat-LyLu cells were transfected with luciferase reporter plasmids containing wild-type CEACAM1 promoter (-194pLuc) or CEACAM1 promoter with mutations in the Sp2 binding site (-194mut pLuc). Fold induction of luciferase activity was calculated relative to that of –194pLuc.



Association of Sp2 with CEACAM1 Promoter *In Vivo*

We further investigated whether Sp2 is associated with the CEACAM1 promoter in vivo by using a chromatin-immunoprecipitation (ChIP) assay (33, 34). Following sonication, chromatin prepared from Mat-LyLu or NbE cells were immunoprecipitated with anti-Sp2 antibody to examine its specific association with the CEACAM1 promoter. Higher levels of Sp2 were found to be associated with the CEACAM1 promoter in Mat-LyLu than NbE cells (Fig. 16). The increase was shown to be ~2.7-fold by quantitative PCR (data not shown). PCR of histone 3.3 was used to show equal chromatin input. This observation suggests that Sp2 is associated with the CEACAM1 promoter in vivo in Mat-LyLu cells.

Fig. 16. Association of Sp2 with CEACAM1 promoter in vivo. ChIP analysis of the CEACAM1 gene in NbE and Mat-LyLu cells was performed using anti-Sp2 antibody. PCR was used to detect nt –162 to –240 region of the CEACAM1 promoter. PCR of histone 3.3 DNA was used as a control for total chromatin input.

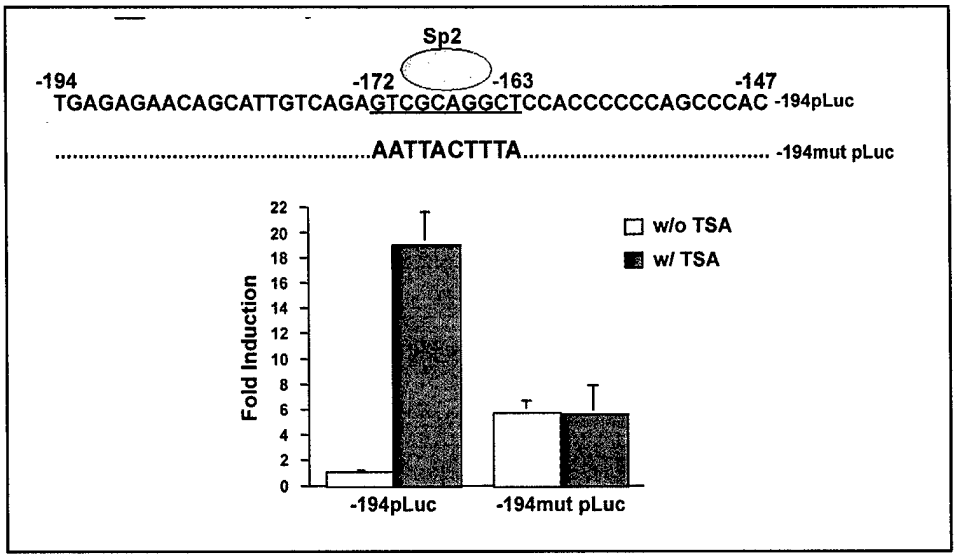


Inhibition of HDAC Activity by TSA Activates CEACAM1 Promoter Activity

The conformation of genes within chromatin determines whether a gene is in its active or inactive state. These structural features are regulated by enzymes that modify chromatin structure. Histone acetylation leads to open chromatin conformation that promotes gene transcription by making promoter sequences accessible to transcription factors. Association with histone deacetylase (HDAC) contributes to the suppressive activity of several transcription factors (35). To investigate whether the inhibitory effect of Sp2 on CEACAM1 promoter activity in Mat-LyLu cells involves the recruitment of HDAC, we employed the HDAC inhibitor trichostatin A (TSA) (36) to examine if TSA can relieve Sp2-mediated repression at the CEACAM1 promoter. Mat-LyLu cells were transfected with -194pLuc or -194mutpLuc and treated with or without TSA (Fig. 17). TSA treatment resulted in about 19-fold increase in the -194pLuc promoter activity, but had little effect on the -194mutpLuc promoter activity. This result suggests that HDAC is involved in the suppression of CEACAM1 promoter activity in Mat-

LyLu cells and the repression requires the presence of a functional Sp2 binding site.

Fig. 17. Inhibition of HDAC activity by TSA activates CEACAM1 promoter activity. Mat-LyLu cells were transfected with –194pLuc or -194mut pLuc in the presence or absence of 1 uM trichostatin A (TSA). Fold induction of luciferase activity was calculated relative to that of –194pLuc in the absence of TSA.

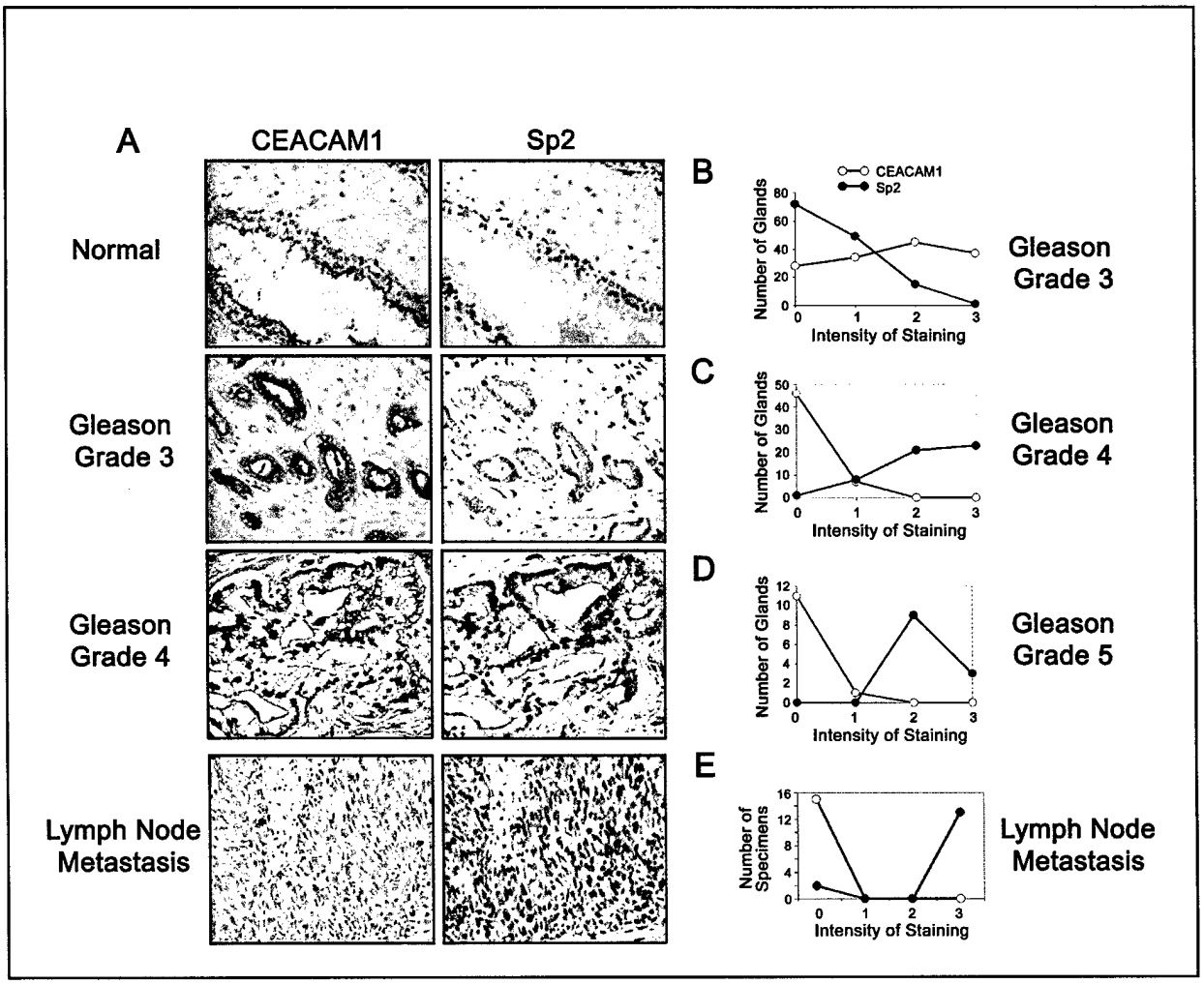


Inverse Relationship Between CEACAM1 and Sp2 Expression in Prostate Cancer Specimens

To address whether the suppressive effect of Sp2 on CEACAM1 expression observed in the prostate cancer cell lines reflects the regulation of CEACAM1 expression in vivo in prostate cancer, the correlation between CEACAM1 and Sp2 expression was determined in consecutive slides from prostate cancer specimens. Studies by Busch et al. (3) showed that down-regulation of CEACAM1 in human prostate cancer occurred at Gleason grade 3 to 4 transition. Using CEACAM1-specific monoclonal antibody Ab 89, CEACAM1 is shown to be expressed in normal and Gleason grade 3 prostate glands but down-regulated in Gleason grade 4 prostate glands (Fig. 18*A*). In contrast, Sp2 was not detected in the normal and Gleason grade 3 glands but was highly expressed in the nuclei of the epithelial cells of Gleason grade 4 prostate glands (Fig. 18*A*). A semi-quantitative assessment of the expression of CEACAM1 and Sp2 in Gleason grade 3 – 5 prostate glands showed an inverse relationship between the expression of CEACAM1 and Sp2 (Fig. 18, *B* – *E*). Metastatic prostate cancer cells in lymph node showed a similar pattern of expression as in Gleason grade 4 and 5 (Fig. 18, *A* and *E*), suggesting that high Sp2 expression concomitant with a loss of CEACAM1 expression occurs in both localized high grade and metastatic prostate cancer cells.

Fig. 18. Expression of CEACAM1 and Sp2 in human prostate cancer specimens. Human prostate

cancer specimens were immunostained with antibodies against CEACAM1 (Ab 89) or Sp2. Representative panels are shown in (*A*) (X 200). In normal prostate gland, CEACAM1 was expressed in the apical surface of epithelial cells while Sp2 was negative. In low-grade prostate cancer (Gleason grade 3), CEACAM1 was expressed in the epithelial cells while Sp2 was negative. In high-grade prostate cancer (Gleason grade 4), fused prostate glands were negative with CEACAM1 staining while strong staining of Sp2 was detected in the nucleus of epithelial cells. In metastatic prostate cancer cells in the lymph node, metastatic prostate cancer cells were completely negative with CEACAM1 staining while they stained strongly with Sp2 in the nucleus. Correlations between Gleason grade and immunostaining score for the expression pattern of CEACAM1 versus Sp2 in prostate cancer specimens are shown in (*B*) – (*E*). The expression levels of CEACAM1 and Sp2 were scored as described in Materials and Methods. *B*, Gleason grade 3 glands (n = 137). *C*, Gleason grade 4 glands (n = 53). *D*, Gleason grade 5 glands (n = 12). *E*, Lymph node metastasis specimens (n = 15). There is an inverse relationship between CEACAM1 and Sp2 expression in prostate cancer specimens.



5.5.4. Discussion

We investigated the mechanism by which CEACAM1 gene expression is down-regulated in prostate cancer. Our studies show that the transcription factor Sp2 is involved in the down-regulation of CEACAM1 in prostate cancer and that this occurs primarily at the level of CEACAM1 gene transcription. Sp2-mediated downregulation of CEACAM1 expression is clinically relevant because the expression pattern of Sp2 is inversely correlated with CEACAM1 expression in prostate cancer cells and in human prostate cancer specimens. Thus, our studies elucidate a novel role for Sp2 as a transcriptional repressor of the CEACAM1 tumor suppressor gene.

5. 6. Studies performed under Task II-1. Determine whether altered chromatin remodeling contributes to CEACAM1 down regulation

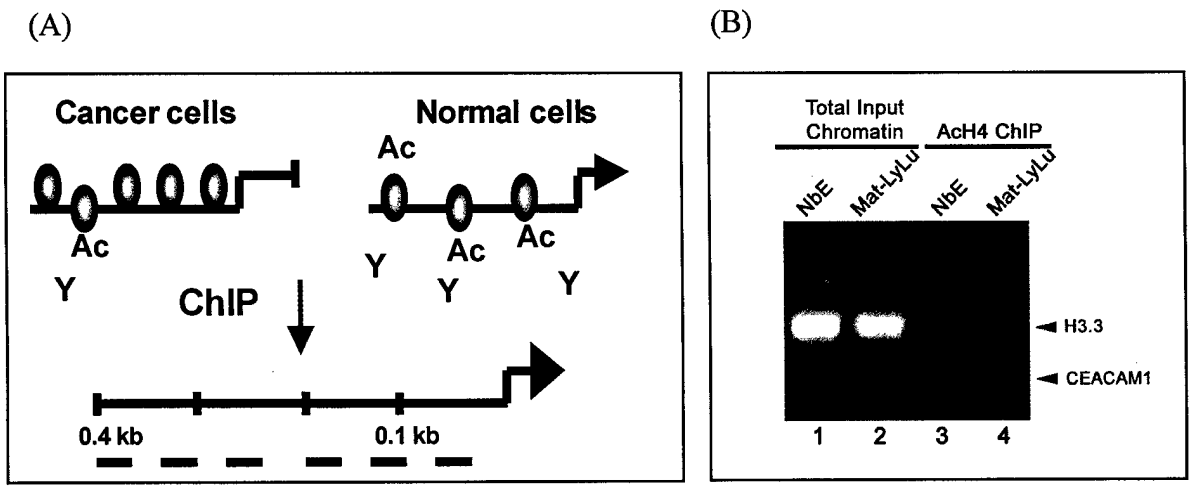
5.6.1. Rationale: The conformation of genes within chromatin determines whether a gene is in its active or inactive state. These structural features are regulated by enzymes that modify chromatin structure. Histone acetylation leads to open chromatin conformation that promotes gene transcription by making promoter sequences accessible to transcription factors. We plan to investigate, using the chromatin immunoprecipitation (ChIP) assay, whether changes in chromatin remodeling might account for CEACAM1 down regulation in tumorigenesis.

5.6.2. Experimental Plan: The ChIP assay will be used to assess whether chromatin remodeling is involved in CEACAM1 promoter inactivation in tumorigenesis. Ac-histone in chromatin can be cross-linked to the associated DNA in vivo. Following immunoprecipitation with anti-acetylated Histone 4 (AcH4) antibody, the associated DNA can be analyzed by polymerase chain reaction (PCR). NbE cells (CEACAM1 expressing normal prostate epithelial cells) and Mat-LyLu (MLL) cells (a metastatic prostate cancer cell line expressing only 4% of CEACAM1 compared with NbE cells) will be analyzed for the extent of the acetylated H4 association with their CEACAM1 promoter. The advantage of the ChIP assay is that chromatin conformation of native promoters can be analyzed. This assay will allow us to address whether histone deacetylases might be involved in transcriptional down-regulation of the CEACAM1 gene in prostate cancer cells and which region of the CEACAM1 promoter might be involved.

5.6.3. Results: Quantitative ChIP assay were carried out as described (33, 34) using the ChIP assay kit (UBI). NbE or Mat-LyLu cells grown on 10 cm culture dishes were treated with formaldehyde to a final concentration of 1% at 37°C for 10 min. The cells were scrape from the plates in phosphate-buffered saline (PBS) containing protease inhibitors. The cell pellets were resuspended in lysis buffer

and sonicated to reduce DNA fragments to 200 to 1000 bp. The cell debris were removed by centrifugation and the supernatant that contained the chromatin were saved for further analysis. Aliquots of these chromatin preparations were used to determine the total DNA content, which was then used to normalize the PCR signals. The remaining chromatin solution was used for immunoprecipitation. Anti-acetyl histone H4 antibody were added to chromatin solution and incubate overnight. The immune complexes were collected with protein-A agarose. The protein-A agarose beads were pellet by centrifugation and the immune complexes eluted by elution buffer. The crosslinked protein-DNA complexes were incubated at 65°C for 4 hours to reverse the crosslink. The proteins were digested with proteinase K and DNA recovered by phenol/chloroform extraction and ethanol precipitation. The genomic DNA fragments bound to anti-acetylated histone antibody were analyzed by quantitative PCR using primer pairs corresponding to different regions of the CEACAM1 promoter. Significant increase (about 2-fold) in the amount of chromatin from the CEACAM1 promoters associated with anti-acetylated histone antibody was observed in the NbE cells compared to that of Mat-LyLu cells (Fig. 19). These results suggest that CEACAM1 promoter region is in more "acetylated" form and thus more "active" state in NbE cells compared to that of Mat-LyLu cells.

Fig. 19. Chromatin remodeling at the CEACAM1 Promoter. (A) Our hypothesis is that more acetylated (Ac) histones are associated with the CEACAM1 promoter in normal prostate epithelial cells, and deacetylation of the histones associated with CEACAM1 promoter may occur during prostate tumorigenesis and give rise to transcriptional down regulation of the CEACAM1 gene. The chromatin immunoprecipitation (ChIP) assay was used to test this hypothesis. (B) PCR of DNA fragments immunoprecipitated by anti-acetylated Histone 4 antibodies. ChIP analysis of the CEACAM1 gene in NbE and Mat-LyLu cells was performed using anti-acetylated histone H4 antibody. PCR was used to detect no –162 to –240 region of the CEACAM1 promoter. PCR of histone 3.3 DNA was used as a control for total chromatin input.



5.6.4. Discussion Our results support the notion that chromatin remodeling is involved in down-regulation of CEACAM1 in tumorigenesis.

(6) KEY RESEARCH ACCOMPLISHMENT

- Chromatin remodeling is involved in the regulation of CEACAM1 expression in prostate tumorigenesis
- AP-2 is one of the transcription activator for CEACAM1 gene expression
- The transcription factor Sp2 plays a role in the down-regulation of CEACAM1 gene in prostate tumorigenesis
- Sp2 recruits histone deacetylase to down-regulate CEACAM1 gene expression
- Androgen receptor up-regulates CEACAM transcription in a ligand-dependent manner. In addition, AR, through its DNA binding domain, directly interacts with CEACAM promoter.

(7) REPORTABLE OUTCOMES

1 manuscript published: Phan, D., Sui, X., Luo, W., Najjar, S., Jenster, G., and **Lin, S.-H.**: Androgen regulation of the Cell-Cell Adhesion Molecule-1 (CEACAM1) gene. Mol. Cell. Endo., **184,** 115-123 (2001).

1 manuscript in press: Phan, D., Tunstead, J., Galfione, M., Luthra, R., Najjar, S. M., Yu-Lee, L.-Y., and Lin, S.-H.: Tumor Specific Regulation of CEACAM1 Gene Expression by Sp2 and Histone Deacetylase. In press in Cancer Research

1 abstract presented at 10th International Workshop of CEA family genes (Sept. 2-5, 1999): Phan, D., Jenster, G., Luo, W., Sui, X., Najjar, S., and Lin, S.-H.: Transcriptional regulation of CEACAM1 gene by androgen receptor.

(8) CONCLUSIONS:

We propose to elucidate the mechanisms that regulate CEACAM1 gene expression in prostate carcinogenesis. We have identified at least two transcription factors, i.e. AP-2 and androgen receptor, that are involved in the up-regulation of CEACAM1 gene expression and one transcription repressor, i.e. Sp2, that specifically down-regulates CEACAM1 promoter activity in tumor cells. In addition, we have developed a novel *in vivo* functional screening method to identify new transcription factors that regulate CEACAM1 gene expression during prostate carcinogenesis. Results from this study will allow us to better understand the regulation of CEACAM1 gene during tumorigenesis and this may

lead to design new therapy strategies to alter tumor progression or to implement early detection and prevention strategies.

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DAMD17-98-1-8465

List of personnel receiving/received pay from the research effort:

Sue-Hwa Lin
Weiping Luo
Karen Earley
Wen-Wei Tsai

(10) APPENDICES:

1 reprint: Phan, D., Sui, X., Luo, W., Najjar, S., Jenster, G., and **Lin, S.-H.**: Androgen regulation of the Cell-Cell Adhesion Molecule-1 (CEACAM1) gene. Mol. Cell. Endo., **184,** 115-123 (2001).

1 manuscript in press in Cancer Research: Phan, D., Cheng, C-J., Galfione, M., Vakar-Lopez, F., Tunstead, J., Thompson, N. E., Burgess, R. R., Najjar, S. M., Yu-Lee, L.-Y., and Lin, S.-H.: Identification of Sp2 as a transcriptional rerpressor of carcinoembryonic antigen-related cell adhesion molecule I in tumorigenesis. Cancer Res. In press.

ELSEVIER

Molecular and Cellular Endocrinology 184 (2001) 115–123

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Androgen regulation of the cell–cell adhesion molecule-1 (*Ceacam1*) gene

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Received 25 January 2001; accepted 26 July 2001

Abstract

Previous studies have established that the cell–cell adhesion molecule-1 (CEACAM1, previously known as C-CAM1) functions as a tumor suppressor in prostate cancer and is involved in the regulation of prostate growth and differentiation. However, the molecular mechanism that modulates CEACAM1 expression in the prostate is not well defined. Since the growth of prostate epithelial cells is androgen-regulated, we investigated the effects of androgen and the androgen receptor (AR) on CEACAM1 expression. Transient transfection experiments showed that the AR can enhance the *Ceacam1* promoter activity in a ligand-dependent manner and that the regulatory element resides within a relatively short (-249 to -194 bp) segment of the 5′-flanking region of the *Ceacam1* gene. This androgen regulation is likely through direct AR-promoter binding because a mutant AR defective in DNA binding failed to upregulate reporter gene expression. Furthermore, electrophoretic mobility shift assays demonstrated that the AR specifically binds to this sequence, and mutation analysis of the potential ARE sequences revealed a region within the sequence that was required for the AR to activate the *Ceacam1* gene. Therefore, the regulation of *Ceacam1* gene expression by androgen may be one of the mechanisms by which androgen regulates prostatic function.

Keywords: CEACAM1; Cell adhesion molecule; Androgen receptor; Tumor suppressor; Prostate

1. Introduction

The cell–cell adhesion molecule-1 (C-CAM1), recently renamed CEACAM1 (Beauchemin et al., 1999), is a member of the immunoglobulin supergene family (Lin and Guidotti, 1989; Lin et al., 1991). CEACAM1 is mainly expressed in epithelial cells of many different tissues, including the prostate (Odin et al., 1988). Loss of CEACAM1 expression is an early event in prostate cancer progression (Kleinerman et al., 1995; Pu et al., 1999), suggesting that this molecule may play an important role in prostate tumorigenesis. Consistent with this hypothesis, expression of CEACAM1 in prostate cancer cells can suppress their tumorigenicity in vivo (Estrera et al., 1999; Hsieh et al., 1995; Luo et al., 1999). These observations suggest that CEACAM1 functions as a tumor suppressor in prostate cancer.

The prostate is an androgen-dependent organ, as androgen is the major regulator of prostate development, growth, and secretory function. Induction of prostate involution using androgen ablation is one of the most effective treatments of late-stage prostate cancer. Since CEACAM1 is a tumor suppressor in prostate cancer, it is important to know whether expression of CEACAM1 in the prostate is regulated by androgen.

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0303-7207/01/$ - see front matter
PII: S0303-7207(01)00638-4

The rat *Ceacam1* promoter belongs to the GC-rich class of TATA-less promoters (Najjar et al., 1996). Deletion and substitution analyses have revealed that the three proximal Sp1 binding sites are essential for basal transcription of the *Ceacam1* gene. In addition, Najjar et al. (1996) have shown that *Ceacam1* promoter activity is stimulated 2–3-fold by insulin, dexamethasone, and cyclic adenosine monophosphate treatment. However, the effect of androgen on *Ceacam1* promoter activity has not been examined. Therefore, in this study, we examined whether the androgen receptor (AR) regulates *Ceacam1* promoter activity.

2. Materials and methods

2.1. Plasmid constructions

The 5′-flanking region of the rat *Ceacam1* gene was cloned as described previously (Najjar et al., 1996). Nucleotides were numbered relative to +1 at the ATG translation initiation codon and labeled as negative numbers to reflect their position upstream (5′) of the ATG site. Using polymerase chain reaction (PCR), 5′ deletion products (−1609, −439, −249, −194, −147, −131, −124 and −112 bp) of the *Ceacam1* gene were synthesized and subcloned at the *Xho*I and *Hin*dIII sites of the pGL3-BASIC plasmid (Promega, Madison, WI) (Najjar et al., 1996).

The mutants −249pLucARE-1Mut and −249pLucARE-2Mut were generated by site-directed mutagenesis of the −249pLuc vector using PCR. Oligo # 305 (reverse primer; AAGCTTTTCTCTTGGGGAAGA) and oligo # 306 (forward primer; *CTCGAG*ATGTTCTAGAACAATGAACCGAAAAGAGATCCCGCGAAGGATGGGAGGACAGCA) were used as primers to introduce substitutions into the ARE-1 region, while oligo # 307 (forward primer; GCTAGCCCGGG*CTCGAG*AGTCGACAGAACAATGAACCGAAAA) and oligo # 305 were used to introduce substitutions into the ARE-2 region; the sequences that were changed from the wild type are underlined. After these PCR products were sequenced to confirm the mutations, they were subcloned at the *Xho*I and *Hin*dIII sites of the pGL3-BASIC plasmid. The construction of the reporter plasmid harboring two androgen response elements and a TATA box driving the luciferase gene (p[ARE]2-E1b-luc) has been described previously (Jenster et al., 1997).

The human AR cDNA expression vector (pAR_0) was constructed using the simian virus 40 (SV40) early promoter and the rabbit β-globin polyadenylation signal as described previously (Brinkmann et al., 1989). The AR mutant expression vector pAR64, in which the first zinc finger in the AR was disrupted by the replacement of two cysteines with serine and phenylalanine, was constructed as described by Jenster et al. (1993). Additionally, the superactive AR expression vector pcDNA-AR_0p65 was constructed by inserting the *Asp718*-(filled in with the Klenow fragment) and *Sac*II digested fragment of pcDNA-AR_{LBD}-p65 into the *Hpa*I and *Sac*II digested pcDNA-AR_0mcs vector (Sui et al., 1999). This resulted in the generation of a fusion protein containing the wild-type AR fused with the transactivation domain of p65/RelA.

2.2. Culture and transfection of HeLa cells

HeLa human cervical carcinoma cells (American Type Culture Collection, Manassas, VA) were maintained in minimal essential medium supplemented with 10% (v/v) fetal calf serum (FCS). These cells (50 000) were plated in a 12-well plate with 10% (v/v) charcoal-stripped FCS 24 h before transfection. The cells were transfected with 0.3 μg of both luciferase reporter plasmid containing a *Ceacam1* promoter fragment and a receptor plasmid containing either wild-type (pAR_0) or modified AR (pAR64 or pcDNA-AR_0p65) per well using Lipofectin (Life Technologies, Inc., Grand Island, NY) according to the manufacturer's guidelines. About 24 h after transfection, the cells were washed and fed with medium containing charcoal stripped serum with or without R1881 (17α-methyltrienolone; NEN Life Science Products, Boston, MA), and the incubations were continued for an additional 24 h. The cells were then lysed in 200 μl lysis buffer, and the luciferase activity was measured using a luciferase assay system (Promega). The experiments were performed in triplicate.

2.3. Electrophoretic mobility shift assay (EMSA)

EMSA was carried out using a bandshift assay system (Promega). Oligonucleotides having sequences corresponding to the region between −194 to −249 bp of the *Ceacam1* promoter were synthesized by Genosys (Houston, TX) and used as probe. In addition, oligonucleotides containing the AR consensus sequence were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and used as competitors. The plasmid pRSET-GST-AR_{DBD} containing a sequence from the AR DNA binding domain fused to GST was constructed by inserting 0.3 kb of the Klenow-treated *Rsr*II/*Xba*I digested ARDBD fragment from AR126 (Jenster et al., 1995) into the Klenow-treated *Nco*I/*Hin*dIII digested pRSET-GST-SRC782-1139 vector (Spencer et al., 1997). The GST-fusion protein containing the AR DNA binding domain (GST-AR_{DBD}) was expressed and purified from *Escherichia coli* BL21(λDE3), and 100 ng of GST-AR_{DBD} protein was used for EMSA. Purified GST protein was used as a negative control.

2.4. Statistic analysis

Student's t-test was used to examine R1881 effects on different types of mutation. We chose the ratio of promoter activities in the presence and absence of R1881 as a dependent variable to avoid variation in basal activity between different experiments.

3. Results

3.1. Localization of an androgen-responsive region in the Ceacam1 promoter

To examine the effect of AR on *Ceacam1* promoter activity, we first tested cell lines that express AR. Although LNCaP cells, which were isolated from the lymph node metastasis of a prostate cancer patient (Horoszewicz et al., 1983), were shown to express AR, the transfection efficiency in this cell line was very low (data not shown). Another prostatic cell line that express AR is NbE cell. NbE cell is a cell line derived from the ventral prostate of Noble rat and is shown to express AR (Chung et al., 1989). We found that the reporter plasmid containing two androgen response elements (p[ARE]$_2$-E1b-luc) could not respond to R1881 stimulation when transfected into the NbE cells (data not shown). However, this reporter was activated 90–340-folds by R1881 when it was co-transfected with a wild type AR plasmid in the NbE cells (data not shown). This observation suggested that the AR in NbE cells was not functional. The reason for AR dysfunction in NbE cells is not known. Previous studies by Jenster et al. (1995) and Sui et al. (1999) have shown that HeLa cells co-transfected with AR and promoter constructs were suitable for AR related studies. As a result, we chose to use HeLa cells co-transfected with AR for this study.

Ceacam1 promoters with different lengths that were constructed by 5′ deletion were cloned in front of the luciferase gene in the reporter plasmid. Each of these plasmids was transiently cotransfected with the AR expression vector pAR$_0$ into HeLa cells; the reporter plasmid containing two androgen response elements and a TATA box derived from the E1b gene (p[ARE]2-E1b-luc) was used as a positive control. In the absence of the androgen analogue R1881, the 1609 bp *Ceacam1* promoter mediated a 106-fold increase in reporter gene

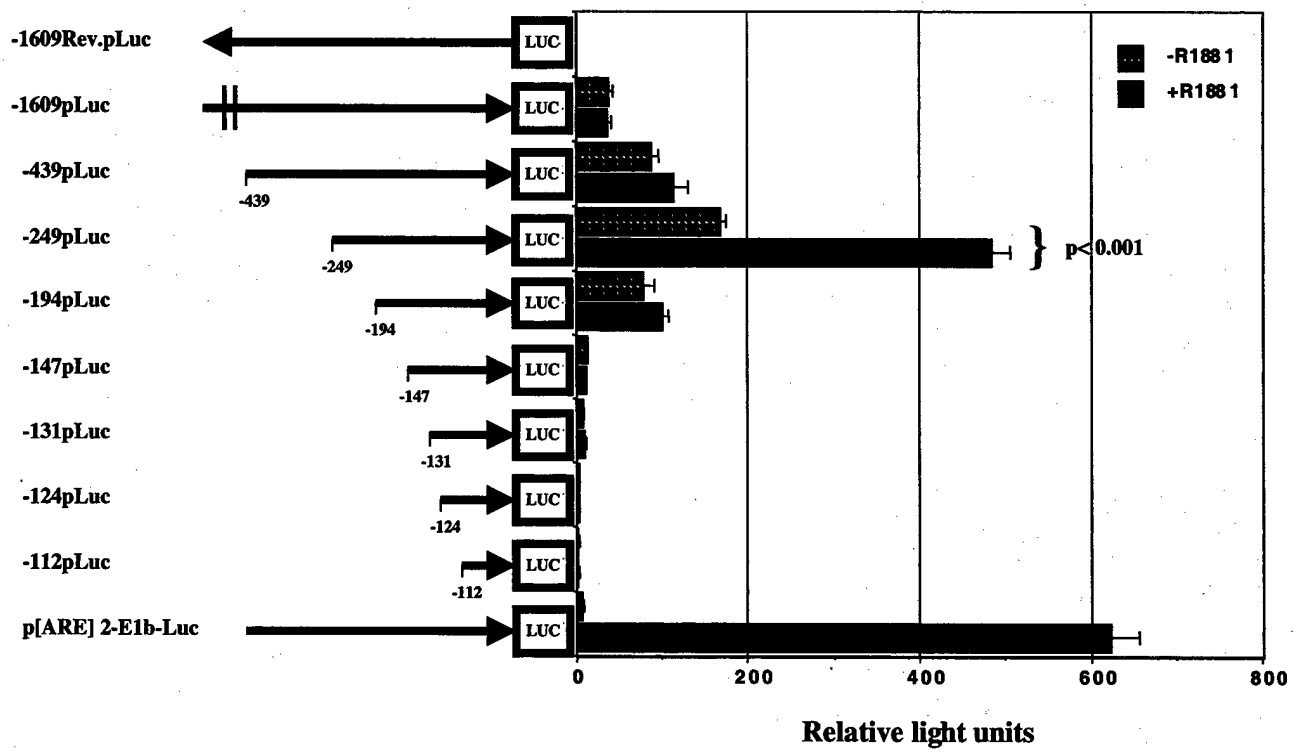

Fig. 1. Regulation of CEACAM1 expression by androgen. A series of reporter plasmids containing *Ceacam1* promoter fragments having different 5′ deletions were cotransfected with a wild-type AR plasmid (pAR$_0$) into HeLa cells. About 24 h after transfection, the cells were incubated with (+) or without (−) 1 nM R1881. The luciferase activity of these cell lysates was determined as described in Section 2. This experiment was repeated eight times with triplicate transfections for each construct and similar results were obtained. Results from one of these experiments were shown and the luciferase activities were reported as the average ± S.D. in relative light units of triplicate transfections. Statistic analysis was used to determine whether there was difference between the R1881 treated and untreated groups. Only −249pLuc construct showed statistically significant difference and the P value for −249pLuc is shown.

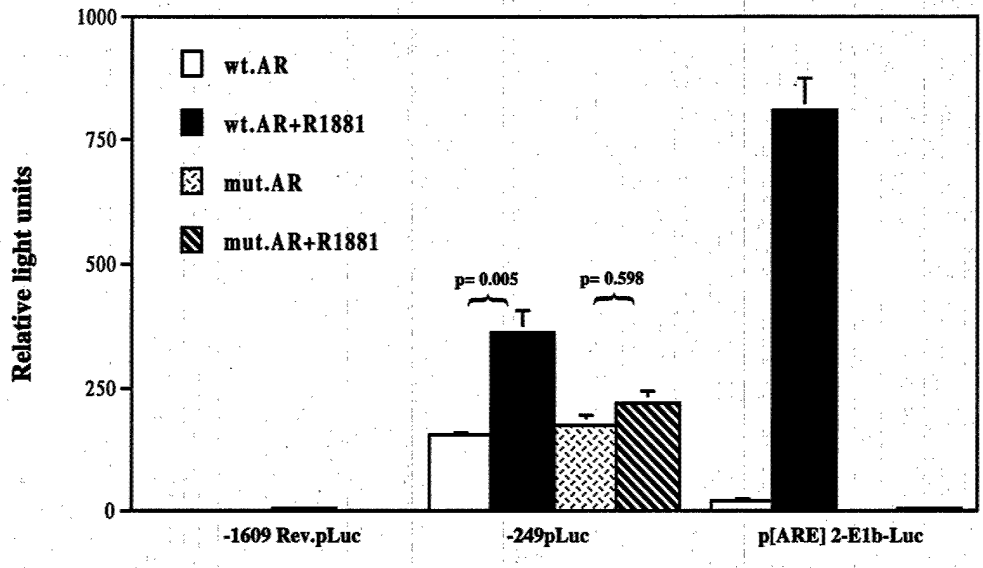

Fig. 2. Effect of an AR mutation on its ability to activate the *Ceacam1* promoter. Cells were transfected with the −249pLuc *Ceacam1* promoter together with a wild-type AR (pAR_0) or mutant AR (pAR64) plasmid, respectively. The data are presented as the mean ± S.E. of three independent experiments. Statistic analysis was used to determine whether there was difference between the R1881 treated and untreated groups. The *P* values for each group are shown.

expression as compared with the reverse-oriented *Ceacam1* promoter fragment (Fig. 1). Deletion of the region between nt −1609 and −439 induced a slight increase in the basal promoter activity (Fig. 1), suggesting that this region may contain potential down-regulators. Deletion of the *Ceacam1* promoter up to −194 bp did not abolish its ability to induce luciferase expression, while deletion up to −147 bp markedly reduced its promoter activity. This result suggested that a minimal promoter is located within the first 194 bp 5′ from *Ceacam1*'s translation start site. We next investigated whether androgen had an effect on the *Ceacam1* promoter. As shown in Fig. 1, the plasmid containing the *Ceacam1* promoter region from −249 to −21 bp exhibited a 2.5-fold increase in luciferase activity upon the addition of the androgen analogue R1881. In contrast, no significant hormone response was observed with plasmids containing the entire 1609, 439, or 194 bp segment proximal to the translation start site. These observations suggested that the region from −249 to −194 bp in the *Ceacam1* gene may contain an androgen-regulated sequence.

3.2. Direct binding of the AR to the promoter sequence

The AR is a 110–112 kDa protein containing transcriptional activation domains in its N-terminal region, a centrally located DNA binding domain, and the ligand binding domain at its C-terminus (Jenster et al., 1991). To test whether activation of the *Ceacam1* promoter by androgen is due to direct interaction between it and the AR, we investigated the effect of a mutant AR, AR64, which is defective in DNA binding (Jenster et al., 1993), on *Ceacam1* promoter activity. In contrast to the wild-type AR, AR64, when cotransfected with −249pLuc into HeLa cells, did not show significant hormone induction (Fig. 2). Similarly, p[ARE]2-E1b-Luc lost its response to R1881 stimulation. These results suggest that activation of the *Ceacam1* promoter by the wild-type AR requires its DNA binding domain; thus, AR may bind directly to *Ceacam1* promoter.

In addition, EMSA was used to determine whether the AR can bind to the promoter sequence. A double-stranded oligonucleotide containing the promoter sequence from −249 to −194 bp was used in the assay. Fig. 3 shows that the AR DNA binding domain can bind to the oligonucleotide (−249 to −194 bp) and that the binding can be specifically competed by the unlabeled corresponding oligonucleotide duplexes, as well as an unlabeled oligonucleotide containing the AR consensus sequence (Roche et al., 1992). This observation suggested that the AR binds specifically to the *Ceacam1* promoter sequence.

3.3. Identification of AR-interacting sites

Using a DNA binding site-selection assay, Roche et al. (1992) determined a consensus AR DNA binding site for the AR. Two regions in the *Ceacam1* promoter, located at −215 to −220 bp and −243 to −248 bp, respectively, showed homology to the consensus half-site sequence and could be responsible for androgen induction of the −249pLuc reporter activity (Fig. 4). These two potential AR binding sites (ARE-1 and ARE-2) were mutated to see if they are indeed involved in androgen regulation. The effect of mutating ARE-1

or ARE-2 on the promoter activity was examined. Mutations of ARE-1 did not cause a significant change in the *Ceacam1* promoter's response to R1881, while mutation of ARE-2 completely abolished the response (Fig. 4). In addition, mutating both ARE-1 and ARE-2 had a similar effect to that of mutating ARE-2 alone. These observations suggested that only ARE-2 is involved in the androgen regulation of *Ceacam1* promoter activity.

A mutational analysis of potential ARE sites was also performed using a superactive AR containing the AR fused with the transactivation domain of p65/RelA (Schmitz and Baeuerle, 1991). As part of the AR_0p65 fusion protein, the p65 activation domain can recruit additional coactivators and proteins of the preinitiation complex resulting in amplification of AR-mediated transcriptional signals. As shown in Fig. 5A, the −249 bp *Ceacam1* promoter activity showed a 5–6-fold increase in response to R1881 stimulation with the superactive AR in contrast to a 2–3-fold increase in response to R1881 stimulation with the wild-type AR. Such an enhancement of reporter activity was used to further confirm the mutational analysis. In the presence of the superactive AR, mutation of ARE-1 resulted in a 4-fold increase in luciferase activity in response to R1881. As observed with wild-type AR, R1881 treatment did not increase the promoter activity of the ARE-2 mutant or combined ARE-1/ARE-2 mutant. These observations further confirmed that the AR only requires ARE-2 to stimulate *Ceacam1* promoter activity.

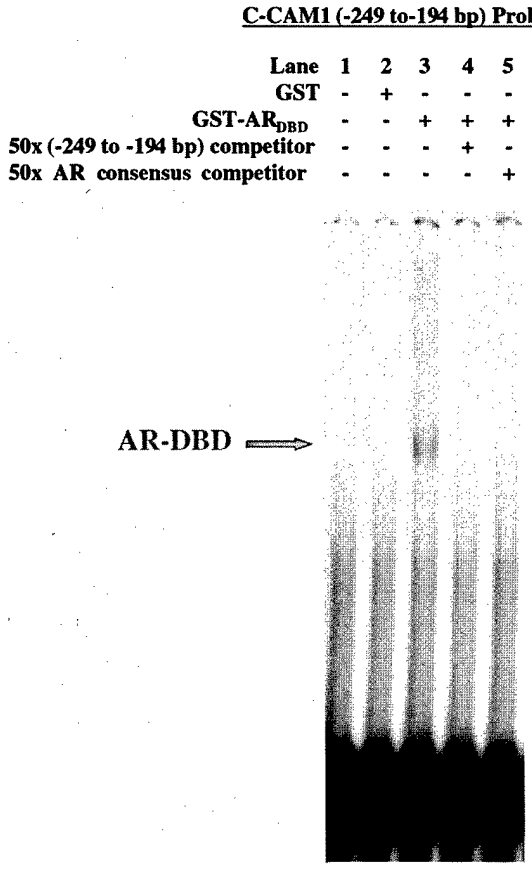

Fig. 3. EMSA, which was carried out using purified GST-AR_{DBD} and the labeled double-stranded oligonucleotide probe containing a sequence from −249 to −194 bp of the *Ceacam1* promoter. Lane 1, without protein; lane 2, with GST protein; lane 3, with GST-AR_{DBD}; lane 4, with GST-AR_{DBD} and a 50-fold molar excess of the unlabeled probe; lane 5, with GST-AR_{DBD} and a 50-fold molar excess of a double-stranded oligonucleotide containing the AR consensus sequence (Roche et al., 1992).

4. Discussion

Androgen is the most important factor that regulates prostate growth and differentiation. A series of genes that have functions related to cell-growth modulation have been shown to be regulated by androgen in prostate cells. It was shown that androgen can directly or indirectly upregulate growth factors such as epidermal growth factor (Hiramatsu et al., 1988; Nishi et al., 1996), keratinocyte growth factor (Fasciana et al., 1996; Peehl and Rubin, 1995; Rubin et al., 1995; Yan et al., 1992), and basic fibroblast growth factor (Katz et al., 1989; Zuck et al., 1992), leading to epithelial-cell proliferation. In addition, transforming growth factor β, which has been linked to programmed cell death, is induced upon androgen withdrawal (Kyprianou et al., 1990). Regulation of growth hormones and apoptotic factors may contribute to the growth of the prostate. On the other hand, androgen upregulation of insulin-like growth factor binding proteins (IGFBP) could make the potent prostate mitogens IGF-I and IGF-II unavailable for growth induction (Gregory et al., 1999). Cell-cycle regulatory proteins such as cdk2, cdk4, cyclin D3, cyclin A, p21CIP1/WAF-1, p27kip1, and p16 were also found to be regulated by androgen (Gregory et al., 1998; Knudsen et al., 1998; Kokontis et al., 1998; Lu et al., 1999, 1997). These diverse androgen-regulated events result in the maintenance of prostate homeostasis; disruption of these intricately balanced events may lead to prostate cancer initiation and progression.

In the present study, we showed that *Ceacam1*, a tumor suppressor gene, can, under defined circumstances and/or in a specific cellular context, be regulated by androgen. Specifically, androgen could up-regulate CEACAM1 expression in a ligand-dependent manner when tested in vitro. This androgen regulation is controlled by only one of the two half-sites of the AR consensus sequence (Roche et al., 1992). A similar event was also observed by Dai and Burnstein (1996), who showed that the presence of one half-site of the AR consensus sequence is sufficient to upregulate the promoter of the AR gene by the AR. This half-site interaction may not provide as strong an activity as that provided by the full consensus sequence in the

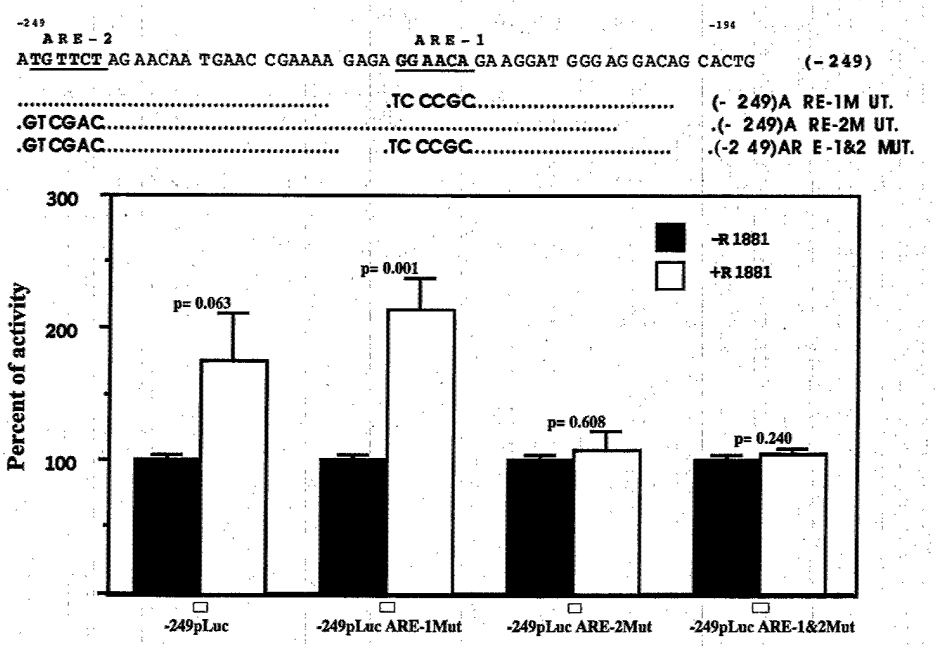

Fig. 4. Mutation analysis of the putative AREs in the *Ceacam1* promoter. The nucleotide sequence from −249 to −194 bp in the rat *Ceacam1* promoter is shown. Two half-sites of putative androgen-responsive elements (ARE-1 and ARE-2) are underlined; the mutated sequence of the two elements is shown. Cells were transfected with reporter plasmids containing the −249 bp promoters, whose putative ARE-1 or ARE-2 sequence was mutated as described in Section 2. The luciferase activity is presented as a percent of that of the corresponding plasmid containing no mutation and without R1881 treatment. The data are presented as the mean ± S.E. of three independent experiments. Statistic analysis was used to determine whether there was difference between the R1881 treated and untreated groups. The *P* values for each group are shown.

probasin promoter (Kasper et al., 1994) and PSA promoter (Zhang et al., 1997). In addition, the longer *Ceacam1* promoter, i.e. the 439-bp segment, did not show ligand-dependent regulation by androgen, suggesting that other regulatory mechanisms may be present upstream of the promoter region and may influence the interaction of the AR with the *Ceacam1* proximal promoter region. Thus, the regulation of CEACAM1 expression by the AR is complex and may depend on its cellular context. In this regard, it is interesting to note that in the rat, CEACAM1 is mainly expressed in the dorsal lobe but not the ventral lobe of the prostate, although the AR is expressed in both prostate lobes (Makarovskiy et al., 1999). In addition, using castration-induced prostate involution followed by administration of androgen, we have previously shown that expression of CEACAM1 in the rat ventral prostate was negatively regulated by androgen in vivo, while its expression in the dorsal prostate showed no response to androgen manipulation in vivo (Hsieh and Lin, 1994; Makarovskiy et al., 1999). In the mouse prostate, on the other hand, increased CEACAM1 expression was detected in the dorsal prostate lobe following castration (Pu et al., 1999). In the present study, we found that androgen could up-regulated CEACAM1 expression in a ligand-dependent manner, although up-regulation of CEACAM1 expression by androgen was observed only with a relatively short promoter (bp −294 to −197). Together, these observations suggest that the regulation of CEACAM1 expression by androgen in vivo is a complex process. Although the AR has a positive effect on the proximal region of the *Ceacam1* promoter, it may have a negative effect on the other region of the *Ceacam1* promoter. AR regulation of CEACAM1 expression is, therefore, influenced by the cellular context and possibly the stromal components of the prostate. The interactions between prostatic epithelial and stromal components have been shown to contribute to androgen-dependent regulation of prostate growth and differentiation (Chung, 1995; Cunha et al., 1987). As a result, although direct interactions between AR and *Ceacam1* promoter may contribute to androgen regulation of CEACAM1 expression, other factors including signals from stromal-epithelial interactions may also influence CEACAM1 expression in vivo.

Primary prostate cancers are largely dependent on androgens for growth and survival. Therefore, the disease responds favorably to androgen ablation and antiandrogen therapy in most patients. However, virtually all patients will relapse with clinically defined androgen-independent cancer. Previously, the role of CEACAM1 in androgen-dependent and androgen-independent prostate cancer was studied by examining the patterns of CEACAM1 expression during prostate cancer initiation, progression, and metastasis in the transgenic adenocarcinoma of mouse prostate (TRAMP) model, which was generated by using the rat probasin pro-

moter to target the SV40 large T antigen specifically to the mouse prostate (Greenberg et al., 1995). In the TRAMP mice, immunohistochemical staining using polyclonal antibody Ab669 against CEACAM1 revealed that the CEACAM1 protein was expressed in normal prostate epithelia, as well as low-grade prostate intraepithelial neoplasia (PIN); the expression was uniform on the luminal surfaces of these epithelia. CEACAM1 expression was noticeably reduced and the staining pattern was heterogeneous in some cases of high-grade PIN, and CEACAM1 staining was generally absent from prostate cancer and metastatic lymph nodes. Androgen-independent prostate cancer and its metastases generated in castrated TRAMP mice were also CEACAM1 negative (Pu et al., 1999). Since loss of CEACAM1 expression occurred before the development of androgen-independent tumors, it is likely that the AR regulation of CEACAM1 expression is not related to the loss of CEACAM1 during prostate cancer progression.

Other factors that have been shown to have an effect on the *Ceacam1* promoter include the upstream stimu-

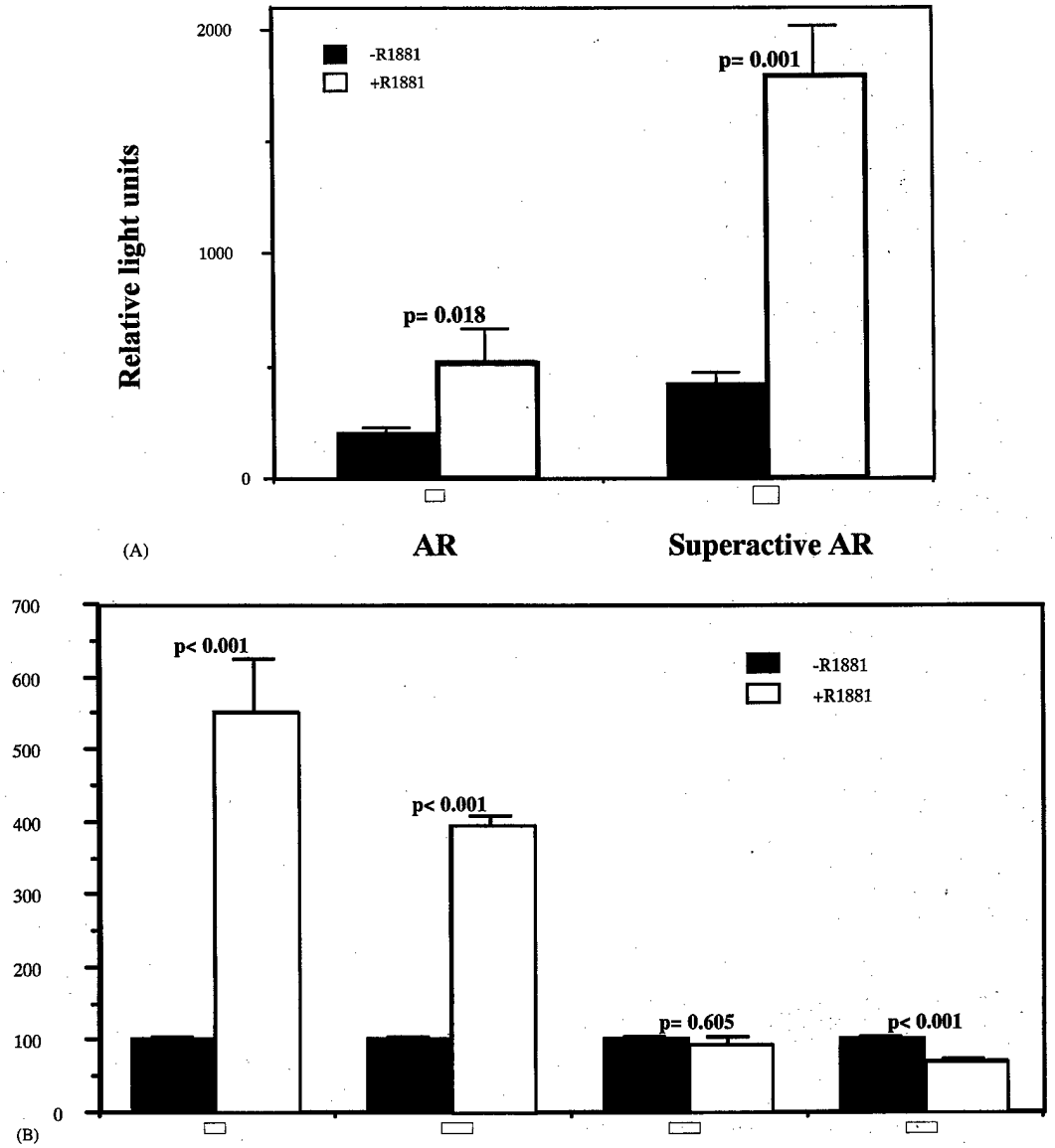

Fig. 5. Activation of *Ceacam1* promoter activity by a superactive AR (AR_0p65). (A) The *Ceacam1* promoter transcription activity was examined using cotransfection of the *Ceacam1* promoter reporter construct (− 249pLuc) and the wild-type or superactive AR expression plasmid (pAR_0p65) into HeLa cells. The luciferase activity was determined from cell lysates of transfected cells as described in Section 2. This experiment was repeated six times with triplicate transfections for each construct and similar results were obtained. Results from one of these experiments were shown and the luciferase activities were reported as the average ± S.D. in relative light units of triplicate transfections. (B) Effect of superactive AR on mutant *Ceacam1* promoter transcription activity. The luciferase activity is presented as a percent of the luciferase activity without R1881 treatment. The data are presented as the mean ± S.E. of three independent experiments. Statistic analysis were performed as described in Section 2 to compare the R1881-treated and untreated groups for each construct, and the *P* values for each group are shown.

latory factor and hepatocyte nuclear factor-4 (Hauck et al., 1994). Also, Chen et al. (1996) showed that treatment of HT-29 cells with interferon-γ (IFN-γ) upregulated CEACAM1 expression. This was due to the ability of IFN-γ to upregulate the expression of IRF-1, which, by binding to the interferon stimulated response element located in the human *Ceacam1* promoter, activated *Ceacam1* transcription. Thus, the regulation of CEACAM1 expression is a combination of different transcriptional factors, one of which is the AR.

Acknowledgements

This work was supported by grants CA64856 from the National Institutes of Health and DAMD17-98-1-8465-1 from the U.S. Department of the Army and predoctoral training grant T32 CA67759 from the National Institutes of Health to Dillon Phan The nucleotide sequences used in this paper are in the GenBank/EMBL Data Bank with accession number U27207 for the 5′-flanking region of the rat CEACAM1/pp120 gene.

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[CANCER RESEARCH 64, ●●●–●●●, May 1, 2004]

Identification of Sp2 as a Transcriptional Repressor of Carcinoembryonic Antigen-Related Cell Adhesion Molecule 1 in Tumorigenesis

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ABSTRACT

Down-regulation of carcinoembryonic antigen-related cell adhesion molecule 1 (*CEACAM1*) tumor suppressor gene expression is common in several malignancies including prostate, colon, and breast cancer. The mechanism that mediates this down-regulation is not known. Here, we report that down-regulation of CEACAM1 expression in prostate cancer cells occurs primarily at the transcriptional level and is mediated by Sp2, a member of the Sp family of transcription factors. Sp2 binds to the CEACAM1 promoter *in vitro* and *in vivo*, and transient overexpression of Sp2 down-regulates endogenous CEACAM1 expression in normal prostate epithelial cells. Sp2 appears to repress *CEACAM1* gene expression by recruiting histone deacetylase activity to the CEACAM1 promoter. In human prostate cancer specimens, Sp2 expression is high in prostate cancer cells but low in normal prostate epithelial cells and is inversely correlated with CEACAM1 expression. Our studies show that transcriptional repression by Sp2 represents one mechanism by which *CEACAM1* tumor suppressor gene is down-regulated in prostate cancer.

INTRODUCTION

Loss of tumor suppressors is one of the major mechanisms that lead to tumor formation. Although tumor suppressors were originally identified as genes whose deletions or mutations cause cancer, their down-regulation occurs more commonly in tumorigenesis (1). Sager *et al.* (1) thus classified the former as type I and the latter as type II tumor suppressors.

Carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), a member of the CEA family, is a M_r 105,000 glycoprotein originally identified as a protein that mediates intercellular adhesion (2). Down-regulation of CEACAM1 was observed in many tumor types including prostate (3, 4), colon (5), endometrium (6), breast (7, 8), and hepatocellular (9) carcinomas, suggesting that CEACAM1 may have an important role in the maintenance of normal epithelial phenotype. In experimental tumor models, reduction of CEACAM1 levels in normal rat prostate NbE cells promoted tumorigenesis (10), whereas re-expression of CEACAM1 in prostate cancer cells suppressed their tumorigenicity *in vivo* (10). Suppression of tumorigenicity by CEACAM1 was also observed in breast (11), bladder (12), and colon carcinoma (13). In addition, the human (14), rat (10), and mouse homologues of CEACAM1 (13) were all shown to have tumor-suppressive activity. These results support the role of CEACAM1 as a tumor suppressor.

The mechanism of CEACAM1-mediated tumor suppression is through inhibition of tumor angiogenesis. Volpert *et al.* (15) showed that conditioned medium from CEACAM1-expressing cells inhibited endothelial cell migration *in vitro* and corneal angiogenesis *in vivo*. This inhibitory effect is due to induction of apoptosis in endothelial cells (15). Thus, it is likely that expression of CEACAM1 in tumor cells induces the production of inhibitory factors that affect tumor angiogenesis, leading to suppression of tumor growth *in vivo*.

The mechanism by which CEACAM1 is lost during tumorigenesis is not clear. Allelic loss of *CEACAM1* gene, localized at chromosome 19 in humans (16) and 7 in mouse (17), has not been reported to occur in either prostate or colon cancer. Although an extensive analysis on human prostate cancer specimens has not been performed, studies by Rosenberg *et al.* (18) using tissues or cells from mouse colon carcinoma showed that neither chromosomal rearrangements nor gene deletions occurred close to the *CEACAM1* gene. Thus, it is likely that down-regulation rather than irreversible loss of CEACAM1 expression is the major cause of tumorigenesis *in vivo*.

In this study, we identify for the first time a mechanism for the loss of CEACAM1 expression in prostate cancer. We found that down-regulation of CEACAM1 expression in prostate tumors occurs mainly at the transcriptional level. In addition, we provide evidence that down-regulation of *CEACAM1* gene in prostate cancer is mediated by the transcription factor Sp2 that is highly expressed in prostate cancer cells. Furthermore, Sp2 recruits histone deacetylase (HDAC) to repress transcription of the CEACAM1 gene. Thus, loss of *CEACAM1* tumor suppressor gene expression in prostate cancer cells is attributed to aberrant chromatin acetylation. Extensive immunohistochemical analysis showed that there is an inverse relationship between CEACAM1 and Sp2 expression in human prostate cancer specimens, providing strong support that high Sp2 levels correlate with the loss of CEACAM1 expression during tumorigenesis.

MATERIALS AND METHODS

Western and Northern Blot Analyses. For Western blot analysis, cells were lysed, and protein concentrations were determined by Coomassie Blue Plus (Pierce). Equal amounts of protein were resolved on SDS-PAGE and immunoblotted with anti-CEACAM1 antibody Ab669 (19). For Northern blot analysis, total cellular RNA (20 μg), prepared by using RNAzolB (Biotecx), was resolved on a 1% agarose gel containing 0.02% formaldehyde and hybridized with a CEACAM1 cDNA probe (20). 36B4 was used as a control for RNA loading (21).

CEACAM1 Promoter Plasmid Construction. The 5′-flanking region of rat *CEACAM1* gene was cloned as described previously (22). Using PCR, 5′ CEACAM1 promoter deletion products (−1609, −439, −249, and −194 bp) were generated and subcloned into the *Xho*I and *Hin*dIII sites of pGL3-BASIC (22). The −194pLuc vector was used in site-directed mutagenesis of the putative Sp2-binding site (underlined) by using Oligo 309 (forward primer, 5′-CTCGAGTGAGAGAACAGCATTGTCAGAAATTACTTTACCACCCCCCAGCCCA) and Oligo 304 (reverse primer, 5′-AAGCTTCTTCTCTTGGGGAAGAGAT). The Sp2 site mutation in −194mut pLuc was confirmed by sequencing.

Transfection of NbE and Mat-LyLu Cells. NbE (23) and Mat-LyLu (24) cells were maintained in DMEM medium supplemented with 10% FCS, plated at 50,000 cells/well in a 12-well plate, and transfected with 0.3 μg plasmid/well using LipofectAMINE (Life Technologies, Inc.). After 24 h, cells were

Received 11/30/03; accepted 2/23/04.

Grant support: NIH Grants DK 54254 and DK 57497 (S. Najjar), CA64856 and CA86342 (S-H. Lin), and CA60896 (R. Burgess); and U.S. Department of the Army Grants DAMD17-98-1-8465-1 and DAMD17-1-00-0031 (S-H. Lin).

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DOI: 10.1158/0008-5472.CAN-03-3730

lysed in 200 μl of lysis buffer, and the luciferase activity was measured (Promega). Where indicated, 5 h after transfection, cells were incubated with 1 μg/ml trichostatin A (TSA; Sigma) for 24 h before luciferase activity was determined. Samples were assayed in triplicate, and each experiment was performed at least three independent times.

Electrophoretic Mobility Shift Assay (EMSA). Ten μg of nuclear proteins were subjected to EMSAs using a bandshift assay system (Promega). Double-stranded oligonucleotides corresponding to −147 to −194 bp (194probe or 194mut probe) of the CEACAM1 promoter were synthesized (Sigma/Genosys) and used as probes. For supershift analysis, nuclear extracts were incubated with 1 μl of rabbit polyclonal antibody specific for Sp1, Sp2, Sp3, or Sp4 (Santa Cruz Biotechnology).

Immunostaining. Cells were fixed in formalin, blocked with normal goat serum for 30 min, and incubated with Sp2 (K-20) antibody (Santa Cruz Biotechnology) at 4°C overnight. Antibody binding was detected by using the ABC kit with 3,3′-diaminobenzidine as the chromogen (Vector). The immunostained cells were then counterstained with hematoxylin.

Chromatin Immunoprecipitation (ChIP) Assay. Quantitative ChIP assays were carried out as described (25, 26) using the ChIP assay kit (Upstate Biotechnology). Chromatin prepared from NbE or Mat-LyLu cells (10-cm dish) was used to determine total DNA input and for overnight incubation with either anti-acetyl histone H4 (Anti-AcH4) antibody (06-866; UBI) or Sp2 antibody. Primers corresponding to nucleotide (nt) −162 to −240 of the CEACAM1 promoter were used in both PCR and quantitative real-time PCR analyses: CEACAM1 forward, 5′-AACAATGAACCGAAAAGAGAGGAA (nt −240 to −217); CEACAM1 reverse, 5′-GAGCCTGCGACTCTGACAATG (nt −183 to −163); and CEACAM1 TaqMan probe, 5′-GTTCTCTCAGTGCTGTCCTCCCATCCTTCT (nt −215 to −186; Perkin-Elmer). Histone 3.3 was used as control for total chromatin: H3.3 forward, 5′-GCAAGAGTGCGCCCTCTACTG; and H3.3 reverse, 5′-GGCCTCACTTGCCTCCTGCAA. Data are representative of four independent experiments.

Immunohistochemistry of Prostate Cancer Specimens. Formalin-fixed, paraffin-embedded tissue samples representing a spectrum of localized and metastatic prostate cancer, including radical prostatectomy specimens and lymph nodes with prostate cancer metastases, were selected from the Prostate Specialized Program of Research Excellence Tissue Bank at The University of Texas M. D. Anderson Cancer Center. A CEACAM1-specific monoclonal antibody (Ab 89) was produced by injecting BALB/c ByJ mice (The Jackson Laboratory, Bar Harbor, ME) with full-length human CEACAM1, which was expressed and purified from Sf9 cells as described previously (27). Serum samples, obtained by supraorbital bleedings, showed high titers (>1:3200) to both CEACAM1 and the closely related CEA (28) by ELISA. Hybridomas were prepared by standard procedures (29). Monoclonal antibodies were screened by ELISA, using CEACAM1 as the antigen, and then cross-screened with human CEA (DAKO, Carpinteria, CA) as the antigen. Ab 89 reacted strongly with CEACAM1, but did not react with CEA. Four-μm-thick sections were dewaxed with xylene, rehydrated in graded alcohol, treated with 3% H_2O_2 in methanol for 15 min, washed with PBS, blocked with normal goat serum for 30 min, and incubated at 4°C overnight with Ab 89 at 1:1000 dilution. For the detection of Sp2, antigen retrieval by boiling the slides in 0.01 M sodium citrate (pH 6.0) and 0.1% NP40 for 10 min was performed before incubation with Sp2 antibody at 1:2000 dilution. Antibody binding was detected by using the LSAB kit with 3,3′-diaminobenzidine as the chromogen (DAKO). The sections were then counterstained with hematoxylin. AQ: D

A scoring system was devised to determine the relative expression of CEACAM1 and Sp2 in the glands of the prostate cancer specimens. For CEACAM1, 0 represents no staining; 1, weak apical staining in part of the gland; 2, variable intensity apical staining in the entire gland; and 3, strong apical staining in the entire gland. For Sp2, 0 represents no staining in both nucleus and cytoplasm; 1, weak cytoplasmic staining; 2, weak cytoplasmic and nuclear staining; and 3, strong nuclear staining. The malignancy of the tumor was graded 1–5 according to Gleason (30).

RESULTS

CEACAM1 Expression in Normal and Prostate Carcinoma Cell Lines. To study the regulation of *CEACAM1* gene during prostate tumorigenesis, we used the Dunning rat prostate cancer cells AT-2, AT-3, and Mat-LyLu to determine whether CEACAM1 protein expression levels show distinct tumor-specific down-regulation. These cell lines represent tumors ranging from relatively benign, slowly growing, differentiated, androgen-sensitive tumors to rapidly growing, anaplastic, hormone-insensitive malignant tumors (24, 31). A prostate cell line NbE, derived from ventral prostate of Noble rat (23), was used as a normal control. A significant decrease in CEACAM1 protein levels occurred in AT-2, AT-3, and Mat-LyLu cells by comparison with NbE normal controls (Fig. 1*A*). In Mat-LyLu cells, CEACAM1 level was about 4% that of NbE normal controls. These results are consistent with our previous observation of reduced CEACAM1 levels in human prostate cancer cells (14) and in mouse prostate cancer tissues (3). Reduced CEACAM1 protein levels are associated with a significant decrease in the steady-state levels of the 4-kb CEACAM1 message in these prostate cancer cells (Fig. 1*B*). These observations indicate that loss of CEACAM1 protein in prostate cancer cells is due to a reduction in the level of the CEACAM1 message. F1

Transcriptional Regulation of *CEACAM1* Gene Expression in Prostatic Cancer Cell Lines. Down-regulation of CEACAM1 expression in prostatic cancer cells could be due to altered transcriptional activity resulting from changes in promoter methylation or the recruitment of factors that attenuate CEACAM1 promoter activity. To distinguish between these possibilities, NbE cells that express CEACAM1 protein and Mat-LyLu cells that have the lowest CEACAM1 level were chosen for additional studies. Treatment of Mat-LyLu cells with the DNA methylation inhibitor 5-aza-2′-deoxycytidine (32) did not result in an increase in CEACAM1 protein expression in Western blot analysis (data not shown). Thus, promoter methylation is unlikely to be involved in human CEACAM1 regula-

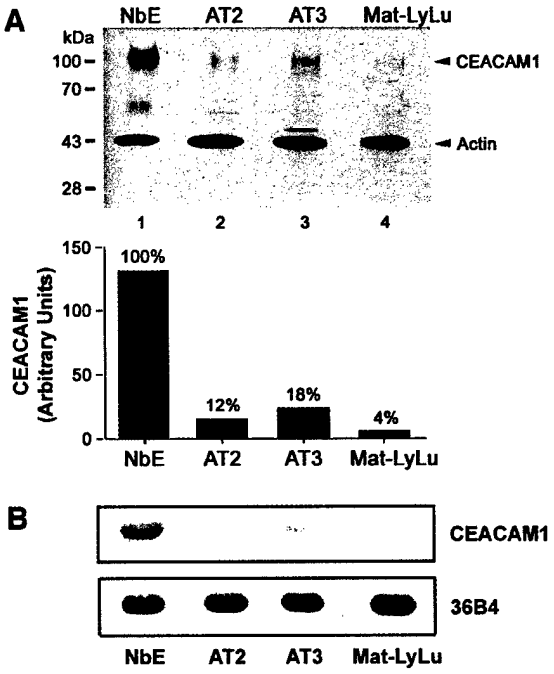

Fig. 1. Down-regulation of CEACAM1 in prostate cancer cells. *A*, immunoblot analysis of CEACAM1 protein expression in NbE and Dunning rat prostate cancer cell lines, using polyclonal anti-CEACAM1 antibody (Ab669). The blot was reprobed with antiactin antibody as a loading control. The intensity of signal from CEACAM1 protein was quantitated by Quantity1 (Bio-Rad). In the Mat-LyLu cell line, the CEACAM1 expression level is about 4% compared with that of normal NbE cells. *B*, Northern blot analysis of CEACAM1 expression. The levels of CEACAM1 messages in NbE, AT2, AT3, and Mat-LyLu cells were tested by Northern blot analysis using a probe generated from the full-length CEACAM1 cDNA (20).

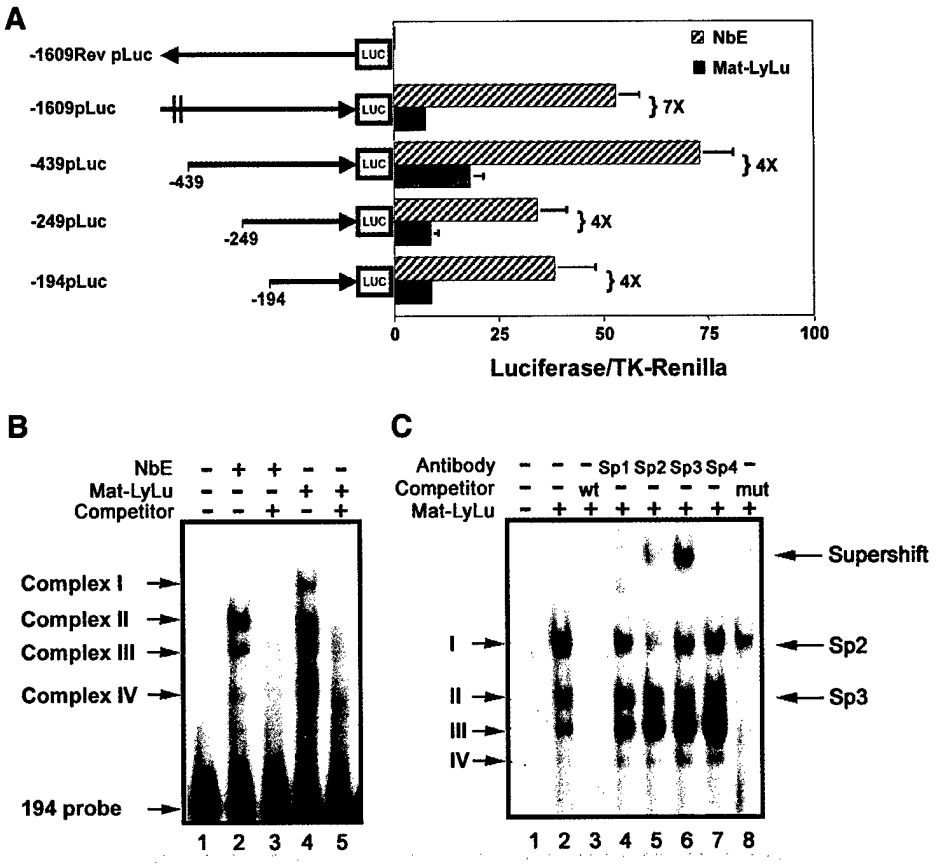

Fig. 2. Comparison of CEACAM1 promoter activities in NbE and Mat-LyLu cells. *A*, a series of reporter plasmids containing various lengths of the CEACAM1 promoter were transfected into NbE or Mat-LyLu cells as described in "Materials and Methods." CEACAM1 promoter (−1609 bp) cloned in reverse orientation (−1609Rev pLuc) was used as a reference. NbE or Mat-LyLu cells transfected with pTK-Renilla were used to normalize the transfection efficiency. After normalization by Renilla luciferase activities, the average luciferase activities ± SD of triplicate transfections were shown. Values located at the *right* of each construct indicate the fold difference in the promoter activity in NbE cells compared with that observed for Mat-LyLu cells. The experiment was repeated three times with triplicate transfections for each construct, and similar results were obtained. Results from one of the experiments are shown. *B*, EMSA of the interaction between NbE or Mat-LyLu nuclear extract with the 194probe. Oligonucleotides corresponding to the region between −194 to −147 bp of the CEACAM1 promoter were synthesized and used as probe (194probe). Nuclear extracts from NbE or Mat-LyLu cells were used. Positions of shifted complexes (complexes I–IV) are indicated by *arrows*. *C*, EMSA in the presence of Sp1, Sp2, Sp3, and Sp4 antibodies. Gel shift analysis using the 194probe was performed in the absence or presence of antibodies against Sp1, Sp2, Sp3, or Sp4 (*Lanes 4–7*). *Lane 4*, anti-Sp1 antibody generated a weak supershifted band, however, no significant decrease was observed in any of the complexes, suggesting that Sp1 might be a minor component in these complexes. *Lane 5*, complex I was shifted by anti-Sp2 antibody as judged by the decrease in complex I intensity. *Lane 6*, complex II was shifted by anti-Sp3 antibody. *Lane 7*, none of the complexes was shifted by anti-Sp4 antibody. *Lane 8*, complex I was not competed by the presence of 100-fold molar excess of the 194mut probe.

tion in prostate cancer, in agreement with the report by Rosenberg *et al.* (18) that methylation was not detected in mouse *CEACAM1* gene in colon carcinoma.

We next examined how transcriptional regulation of *CEACAM1* gene occurs in tumorigenesis. Previous deletion analysis identified a minimal promoter located between nt −194 and −147 proximal to the CEACAM1 translation start site (22, 33). To compare promoter activities in the CEACAM1-positive and -negative cells, we transfected the CEACAM1 promoter-luciferase constructs into normal NbE and Mat-LyLu prostatic cancer cells. A similar pattern of promoter activity was observed in the −1609-bp, −439-bp, −249-bp, and −194-bp CEACAM1 promoter reporter constructs in the Mat-LyLu cells as compared with the NbE cells (Fig. 2*A*). However, there is a reproducible 4-fold decrease in the overall CEACAM1 promoter activity in the fast-growing malignant Mat-LyLu cells. The −147-bp CEACAM1 promoter was inactive in either cell types (data not shown). These results suggest that down-regulation of CEACAM1 in prostate cancer cells is correlated with altered CEACAM1 promoter activity and that a tumor-specific promoter regulatory activity lies between nt −194 and −147 in the minimal CEACAM1 promoter.

Binding of NbE and Mat-LyLu Nuclear Extracts to CEACAM1 Promoter. To identify the tumor-specific activity at the CEACAM1 promoter, we used gel shift assays (EMSA) to examine binding of nuclear proteins from NbE *versus* Mat-LyLu cells to a double-stranded oligonucleotide containing the minimum CEACAM1 promoter (nt −194 to −147, 194probe). Four major bands (complex I–IV) were detected in the Mat-LyLu (Fig. 2*B*, *Lane 4*) nuclear extract. The binding of complex I–IV is specific as it can be competed by an excess of unlabeled 194probe (Fig. 2*B*, *Lane 5*). In contrast, three major bands (complex II–IV), which can be competed by an excess of unlabeled 194probe, were detected in the NbE nuclear extract (Fig. 2*B*, *Lane 2 versus 3*). As complex I is only present in the Mat-LyLu extract, we investigated the possibility that the protein in this complex may play a role in the decreased CEACAM1 promoter activity in Mat-LyLu cells.

Involvement of Sp Transcription Factor Family at the CEACAM1 Promoter. Sequence analysis showed that the 194-bp region of the CEACAM1 promoter is highly GC-rich and contains elements that match the consensus binding sequence for Sp1 (22). To determine whether the Sp family of transcription factors is present at the CEACAM1 promoter, we used antibodies against Sp1, Sp2, Sp3,

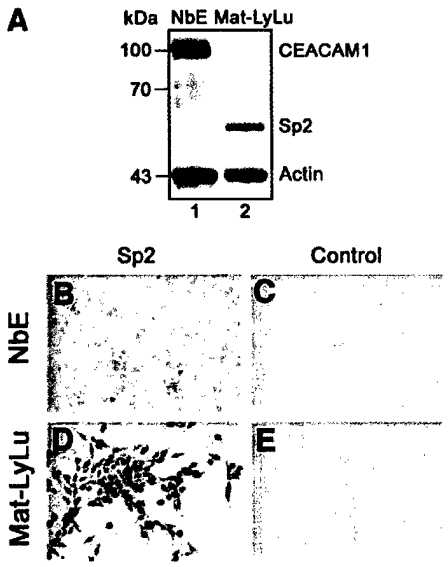

Fig. 3. Sp2 expression correlates with CEACAM1 down-regulation. *A*, differential expression of Sp2 in NbE *versus* Mat-LyLu cells. Cell lysates (20 μg) prepared from NbE or Mat-LyLu cells were boiled in SDS sample buffer, and the proteins were separated by SDS-PAGE. The proteins were transferred onto nitrocellulose membrane and blotted sequentially with anti-Sp2, anti-CEACAM1, and antiactin antibodies. *B*, localization of Sp2 in NbE cells. *D*, localization of Sp2 in Mat-LyLu cells. Mat-LyLu or NbE cells grown on coverslips were fixed by formaldehyde and immunostained with anti-Sp2 antibody. The localization of Sp2 protein was detected by diaminobenzidine. NbE (*C*) or Mat-LyLu (*E*) cells stained with secondary antibody alone were used as controls.

and Sp4 to identify the protein(s) present in complex I in Mat-LyLu cells. Complex I was shifted by anti-Sp2 antibody, as shown by the decrease in complex I intensity (Fig. 2*C*, *Lane 5*). Complex II was shifted by anti-Sp3 antibody (Fig. 2*C*, *Lane 6*). Anti-Sp1 antibody generated a weak supershifted band with no significant decrease in the intensity of any of the complexes, suggesting that Sp1 is a minor component in these complexes (Fig. 2*C*, *Lane 4*). Anti-Sp4 antibody did not generate any supershifted band (Fig. 2*C*, *Lane 7*). Because Sp2 is present in complex I, it is likely that Sp2 is one of the transcription factors that suppresses CEACAM1 promoter activity in Mat-LyLu cells.

Levels of Sp2 Protein in Cells Correlate with CEACAM1 Down-Regulation. Down-regulation of CEACAM1 in Mat-LyLu cells might be due to an increase in the concentrations of Sp2 in Mat-LyLu cells in comparison with NbE cells. Western blot analysis showed that the Sp2 level in Mat-LyLu cells was 10-fold higher than that in NbE cells and is inversely correlated with CEACAM1 levels in these cells (Fig. 3*A*). Furthermore, staining of Sp2 protein was higher in Mat-LyLu than NbE cells by both immunofluorescence (not shown) and immunoperoxidase detection (Fig. 3, *B–E*). Consistent with the role of Sp2 as a transcription factor, immunolocalization analysis revealed stronger nuclear than cytoplasmic staining of Sp2 in Mat-LyLu cells (Fig. 3*D*). Higher levels of Sp2 in Mat-LyLu than NbE cells suggest a potential repressive function of Sp2 on *CEACAM1* gene expression in prostate cancer cells.

Suppression of *CEACAM1* Gene Expression by Sp2. To investigate whether Sp2 has a direct suppressive effect on the CEACAM1 promoter, we transfected NbE cells, which have low to undetectable levels of Sp2 (Fig. 3*A*), with Sp2 expression vector and CEACAM1 promoter reporter construct. Overexpression of Sp2 resulted in a dose-dependent inhibition of CEACAM1 promoter activity as compared with an empty pcDNA3.1 vector control (Fig. 4*A*). This Sp2-mediated suppression was also observed on endogenous CEACAM1 protein levels in Sp2-transfected NbE cells (Fig. 4*B*). These results suggest that Sp2 is one of the factors that mediate a decrease in CEACAM1 expression in Mat-LyLu cells. Taken together, these results are consistent with the interpretation that Sp2 suppresses the expression of CEACAM1 in prostate cancer cells.

Sp2 Binding at the CEACAM1 Promoter. Sp2 is known to bind to GT-box sequences (34, 35), and a GT-box-like sequence is located between nt -172 to -163 at the CEACAM1 promoter. We mutated the GT-box sequences to generate a 194-mut oligo and used it as a competitor in EMSA. Addition of 100-fold excess of the 194-mut oligo blocked complex II–IV formation but did not alter complex I formation (Fig. 2*C*, *Lane 8*). This suggests that Sp2 binds to the GT-rich sequence between nt -172 to -163 in the CEACAM1 promoter. We next generated a -194pLuc reporter containing the Sp2 site mutation (-194mutpLuc) and determined whether the loss of Sp2 binding results in an increase in CEACAM1 promoter activity in Mat-LyLu cells. Mutation of the Sp2-binding site reproducibly resulted in a 6–7-fold increase in CEACAM1 promoter activity in Mat-LyLu cells when compared with that of the wild-type CEACAM1 promoter (Fig. 4*C*). This suggests that binding of Sp2 to nt -172 to -163 of the CEACAM1 promoter suppresses CEACAM1 promoter activity in prostate cancer cells.

Association of Sp2 with CEACAM1 Promoter *in Vivo*. We further investigated whether Sp2 is associated with the CEACAM1 promoter *in vivo* by using a ChIP assay (25, 26). After sonication, chromatin prepared from Mat-LyLu or NbE cells was immunoprecipitated with anti-Sp2 antibody to examine its specific association with the CEACAM1 promoter. Higher levels of Sp2 were found to be associated with the CEACAM1 promoter in Mat-LyLu than NbE cells (Fig. 4*D*). The increase was shown to be ~2.7-fold by quantitative PCR (data not shown). PCR of histone 3.3 was used to show equal chromatin input. This observation suggests that Sp2 is associated with the CEACAM1 promoter *in vivo* in Mat-LyLu cells.

Inhibition of HDAC Activity by TSA Activates CEACAM1 Promoter Activity. The conformation of genes within chromatin determines whether a gene is in its active or inactive state. These structural features are regulated by enzymes that modify chromatin structure. Histone acetylation leads to open chromatin conformation that promotes gene transcription by making promoter sequences accessible to transcription factors. Association with HDAC contributes to the suppressive activity of several transcription factors (36). To investigate whether the inhibitory effect of Sp2 on CEACAM1 promoter activity in Mat-LyLu cells involves the recruitment of HDAC, we used the HDAC inhibitor TSA (37) to examine whether TSA can relieve Sp2-mediated repression at the CEACAM1 promoter. Mat-LyLu cells were transfected with -194pLuc or -194mutpLuc and treated with or without TSA (Fig. 4*E*). TSA treatment resulted in about 19-fold increase in the -194pLuc promoter activity but had little effect on the -194mutpLuc promoter activity. This result suggests that HDAC is involved in the suppression of CEACAM1 promoter activity in Mat-LyLu cells, and the repression requires the presence of a functional Sp2-binding site.

ChIP assays using anti-acetylated histone H4 antibody followed by quantitative PCR showed a ~1.7-fold increase in H4 acetylation at the CEACAM1 promoter in NbE cells than in Mat-LyLu cells (Fig. 4*D*), thus further demonstrating that the CEACAM1 promoter is in a more "active" state in NbE cells than in Mat-LyLu cells. These results agree with the finding that CEACAM1 protein levels are higher in nontumorigenic NbE cells than in the more tumorigenic Mat-LyLu cells. Taken together, these observations suggest that interaction of Sp2 with the CEACAM1 promoter and the subsequent recruitment of HDAC to the CEACAM1 promoter lead to decreased chromatin acetylation at the CEACAM1 promoter, and this results in decreased *CEACAM1* gene expression in Mat-LyLu cells.

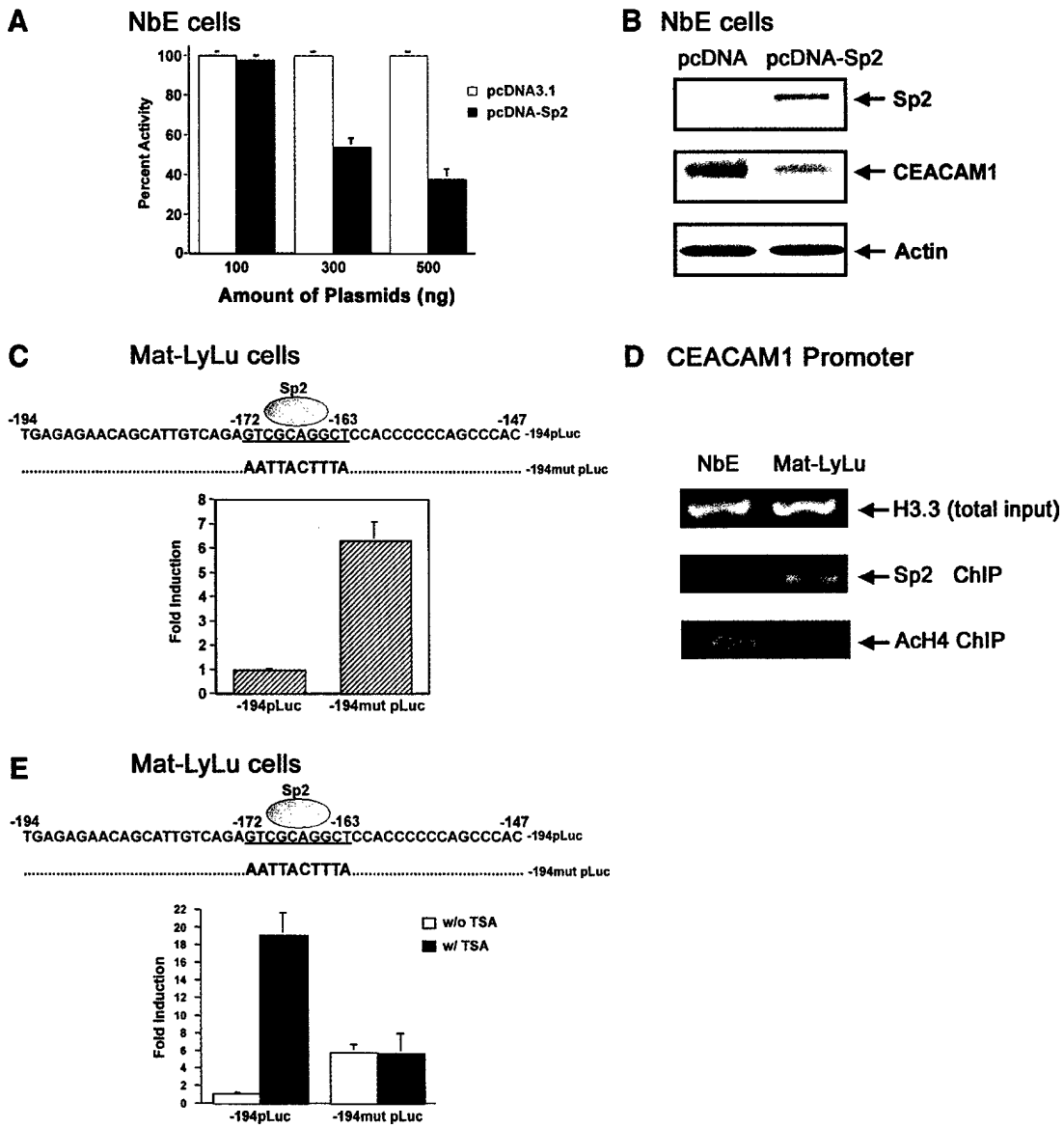

Fig. 4. Suppression of CEACAM1 expression by Sp2. *A*, Sp2 decreases transcriptional activity of CEACAM1 promoter in NbE cells. NbE cells were cotransfected with CEACAM1 promoter reporter plasmid (−1609pLuc) and various amounts of pcDNA-Sp2 expression vector (35) or control vector pcDNA3.1 as indicated. The luciferase activity is presented as a percentage of that of control plasmid-transfected cells. The data are presented as the mean ± SE of three independent experiments. *B*, increased expression of Sp2 inhibited endogenous CEACAM1 expression in NbE cells. NbE cells were transfected with control expression vector pcDNA3.1 or pcDNA-Sp2 expression vector. Twenty-four h after transfection, the cells were harvested and lysed in radioimmunoprecipitation assay buffer. Equal amounts of proteins were loaded and electrophoresed on a 4–12% SDS-polyacrylamide gel. Western immunoblot analysis was performed by using anti-Sp2 antibody and anti-CEACAM1 antibody (Ab669). The expression of actin was used as a control. *C*, mutation of Sp2-binding site increases CEACAM1 promoter activity. Mat-LyLu cells were transfected with luciferase reporter plasmids containing wild-type CEACAM1 promoter (−194pLuc) or CEACAM1 promoter with mutations in the Sp2-binding site (−194mut pLuc). Fold induction of luciferase activity was calculated relative to that of −194pLuc. *D*, association of Sp2 with CEACAM1 promoter *in vivo*. ChIP analysis of the *CEACAM1* gene in NbE and Mat-LyLu cells was performed using anti-Sp2 antibody or anti-acetylated histone H4 antibody. PCR was used to detect nt −162 to −240 region of the CEACAM1 promoter. PCR of histone 3.3 DNA was used as a control for total chromatin input. *E*, inhibition of HDAC activity by TSA activates CEACAM1 promoter activity. Mat-LyLu cells were transfected with −194pLuc or −194mut pLuc in the presence or absence of 1 μM TSA. Fold induction of luciferase activity was calculated relative to that of −194pLuc in the absence of TSA.

Inverse Relationship between CEACAM1 and Sp2 Expression in Prostate Cancer Specimens. To address whether the suppressive effect of Sp2 on CEACAM1 expression observed in the prostate cancer cell lines reflects the regulation of CEACAM1 expression *in vivo* in prostate cancer, the correlation between CEACAM1 and Sp2 expression was determined in consecutive slides from prostate cancer specimens. Studies by Busch *et al.* (4) showed that down-regulation of CEACAM1 in human prostate cancer occurred at Gleason grade 3 to 4 transition. Using CEACAM1-specific monoclonal antibody Ab 89, CEACAM1 is shown to be expressed in normal and Gleason grade 3 prostate glands but down-regulated in Gleason grade 4 prostate glands (Fig. 5*A*). In contrast, Sp2 was not detected in the normal and FS Gleason grade 3 glands but was highly expressed in the nuclei of the epithelial cells of Gleason grade 4 prostate glands (Fig. 5*A*). A semiquantitative assessment of the expression of CEACAM1 and Sp2 in Gleason grade 3–5 prostate glands showed an inverse relationship between the expression of CEACAM1 and Sp2 (Fig. 5, *B–E*). Metastatic prostate cancer cells in lymph node showed a similar pattern of expression as in Gleason grades 4 and 5 (Fig. 5, *A* and *E*), suggesting that high Sp2 expression concomitant with a loss of CEACAM1 expression occurs in both localized high-grade and metastatic prostate cancer cells.

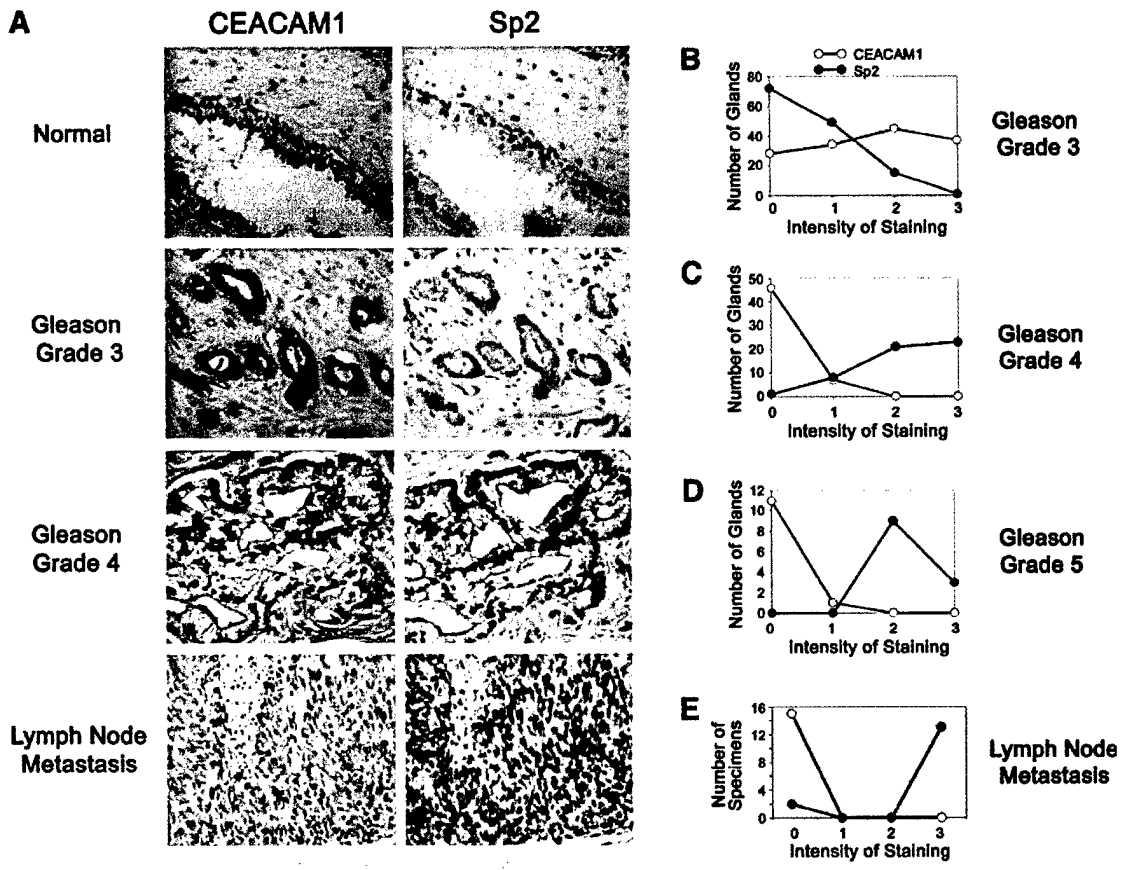

Fig. 5. Expression of CEACAM1 and Sp2 in human prostate cancer specimens. Human prostate cancer specimens were immunostained with antibodies against CEACAM1 (Ab 89) or Sp2. Representative panels are shown in *A* (magnification, ×200). In a normal prostate gland, CEACAM1 was expressed in the apical surface of epithelial cells, whereas Sp2 was negative. In low-grade prostate cancer (Gleason grade 3), CEACAM1 was expressed in the epithelial cells, whereas Sp2 was negative. In high-grade prostate cancer (Gleason grade 4), fused prostate glands were negative with CEACAM1 staining, whereas strong staining of Sp2 was detected in the nucleus of epithelial cells. In metastatic prostate cancer cells in the lymph node, metastatic prostate cancer cells were completely negative with CEACAM1 staining, whereas they stained strongly with Sp2 in the nucleus. Correlations between Gleason grade and immunostaining score for the expression pattern of CEACAM1 *versus* Sp2 in prostate cancer specimens are shown in *B–E*. The expression levels of CEACAM1 and Sp2 were scored as described in "Materials and Methods." *B*, Gleason grade 3 glands (n = 137). *C*, Gleason grade 4 glands (n = 53). *D*, Gleason grade 5 glands (n = 12). *E*, lymph node metastasis specimens (n = 15). There is an inverse relationship between CEACAM1 and Sp2 expression in prostate cancer specimens.

DISCUSSION

We investigated the mechanism by which *CEACAM1* gene expression is down-regulated in prostate cancer. Our studies show that the transcription factor Sp2 is involved in the down-regulation of CEACAM1 in prostate cancer and that this occurs primarily at the level of *CEACAM1* gene transcription. Sp2-mediated down-regulation of CEACAM1 expression is clinically relevant because the expression pattern of Sp2 is inversely correlated with CEACAM1 expression in prostate cancer cells and in human prostate cancer specimens. Thus, our studies elucidate a novel role for Sp2 as a transcriptional repressor of the *CEACAM1* tumor suppressor gene.

Sp2 is a member of the Sp family of transcription factors, which consists of Sp1 through Sp5 (34, 38). Extensive studies have been reported on the functions of Sp1, Sp3, and Sp4 (34). Sp1 is a general transcription factor that regulates many ubiquitously expressed genes by binding to GC boxes at their promoters (34). In contrast, very little is known about the function and expression of Sp2. Sp2 has been shown to bind to a GT-box promoter element within the T-cell receptor α promoter *in vitro* (35). Recently, Sp2 was shown to repress Sp1- and Sp3-mediated activation of the CTP:phosphocholine cytidylyltransferase α promoter in *Drosophila* SL2 cells (39). Although Sp2 expression was detected in several tumor cell lines (35), Sp2 expression in normal tissues has not been examined. In the human prostate specimens, we found that Sp2 expression is low to undetectable in normal epithelial cells but high in prostate cancer cells, suggesting that Sp2 expression is related to tumorigenesis. Sp2 may be involved in tumorigenesis via its inhibition of the *CEACAM1* tumor suppressor gene and/or other growth regulatory genes.

Chromatin remodeling is a fundamental mechanism governing gene regulation during embryonic development, and it also plays a significant role in tumorigenesis. HDAC has an important role in these processes (36, 40). At least 10 HDACs (both class I and II) have been identified in mammalian systems (41, 42). Transcriptional repression through HDACs can arise from a direct interaction of transcription factors with HDACs or indirect interaction via corepressors or adaptors (43, 44). Our observations that CEACAM1 promoter activity was increased by TSA treatment and that the CEACAM1 promoter was hypoacetylated in cancer compared with normal cells suggest that histone deacetylation constitutes a basic mechanism underlying the down-regulation of *CEACAM1* gene expression during tumorigenesis. Co-immunoprecipitation assays indicated that Sp2 does not directly interact with HDAC1 (data not shown). However, it remains possible that Sp2 interacts with other members of the HDAC family or that Sp2 recruits HDAC through corepressors or other adaptor proteins. Additional investigation is required to elucidate this interaction.

In conclusion, our studies suggest that Sp2 mediates the down-regulation of CEACAM1 expression in prostate tumors in part by recruiting HDAC to the CEACAM1 promoter. HDAC, which may be

tethered to the CEACAM1 promoter by Sp2, deacetylates promoter proximal histones and leads to an altered chromatin conformation that prevents transcriptional activation of the *CEACAM1* gene. The identification of Sp2 as a transcriptional repressor raises interesting questions concerning the role of Sp2 in regulating the expression of genes involved in cell growth and differentiation, cancer progression, and tumorigenesis.

ACKNOWLEDGMENTS

We thank Dr. Astar Winoto for providing the Sp2 expression vector, Dr. Leland Chung for providing NbE cells, Dr. Guido Jenster for helpful discussion, and Dr. Raji Luthra for assistance in quantitative PCR.

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